THE EVOLUTIONARY ECOLOGY OF HOST-MICROBIOME SYMBIOSIS IN *ONTHOPHAGUS* DUNG BEETLES

Erik Stetson Parker

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Doctoral Committee

_____

Armin P. Moczek, Ph.D.

_____

Jen Lau, Ph.D.

_____

Jay T. Lennon, Ph.D.

_____

Irene L.G. Newton, Ph.D.

_____

Whitney M. Schlegel, Ph.D.

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Erik Stetson Parker

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The effect of host-microbe interactions on diverse aspects of host biology are increasingly appreciated across biological disciplines, yet the roles played by these interactions in shaping host evolution remain poorly understood. My dissertation research seeks to address these and related issues using the dung beetle genus *Onthophagus*. Previous work in this genus has demonstrated that mothers reliably pass to their offspring a conserved group of gut microbes, and that these vertically inherited microbes enhance offspring growth, development, and survival, especially under stress. In the first three chapters of my dissertation, I employed a manipulative method which allowed for the exchange of gut microbiota between *Onthophagus* species. Using this technique, I was able to first show that different species have diverged to make use of non-interchangeable gut microbiota, and that disruption of these specific host-microbiota relationships has potentially long-term evolutionary consequences. Secondly, I then showed that this host-microbiota species specificity can arise over evolutionarily short timespans, including recently divergent, broadly sympatric and often syntopic sister species sharing virtually identical ecologies. In my third chapter, I was able to show that *Onthophagus* microbiota may influence population adaptation to local thermal conditions. However, contrary to my original hypotheses, results suggested that local host microbiome interactions may limit, rather than enhance, host fitness. Finally, in my fourth chapter, I employed a microbial sequencing approach to provide an in-depth assessment of the taxonomic composition of the gut microbiota of several dung beetle species, and to determine to what extent microbiome composition changes when hosts are

introduced to novel geographic ranges. As a whole, my dissertation employs a diversity of methodologies to better understand the evolutionary and ecological ramifications of dung beetle microbiome symbioses.

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CHAPTER 1

Introduction

OVERVIEW

A major objective of evolutionary biology is to better understand the processes underlying the generation of biodiversity. It has long been accepted that environmental forces acting on developing organisms are strong agents of selection, and that their diverse influences shape adaptive radiations in both plants and animals. Implicit in this view is the notion of an environment, whether biotic or abiotic, that is cleanly separable from and external to a developing organism. However, growing evidence of the ubiquity and importance of the intimate interactions between hosts and their microbial communities has thrown into doubt the continued usefulness of this restrictive definition of the environment. This is evident, for instance, in the common observation that separating a host from its microbiota is detrimental to the fitness of both partners, meaning that host and microbiota reciprocally create critical developmental environments for each other. In this first chapter, I begin by outlining evidence which supports the notion that reciprocal host-microbiota relationships act to structure the microbial environment and explain how this updated view can be used to better address specific questions in the field of evolutionary biology. I then introduce my study system, the dung beetle genus *Onthophagus*, and discuss how it can be leveraged to answer the questions opened up by this new paradigm. Finally, I end with an outline of the specific aims of my dissertation – the general goal of which is to expand our understanding of the evolutionary ecology of microbial symbiosis.

THE MICROBIAL ENVIRONMENT AND ITS ROLE IN ADAPTATION

It is easy to understand how environmental influences could be conceived of as separable and external to an organism. Separable, because how could a tree be expected to change the intensity of the sunlight on any given day? External, because the temperature in any given animals' environment is just that: environmental, and not internal to the animal. But the ability of plants to plastically respond to light cues, and of animals to shift their physiology and behavior to alter their realized environmental exposure are hallmarks of all living organisms. Indeed, nature is full of examples of *biologically relevant* environmental factors being influenced in manifold ways by the organisms who experience them (Moczek, 2015). And among these examples, few serve to challenge both aspects of the separable-external paradigm more thoroughly than host-microbiota interactions. The rise of low-cost, high-throughput sequencing technologies has demonstrated that the microbial symbionts living in and on nearly all organisms exert a stunningly diverse array of influences on their hosts, including (but not limited to) nutritional supplementation (reviewed in Douglas, 2009), life history decisions (such as metamorphosis induction: Hadfield, 2011; Shikuma et al., 2014; reproductive timing: Leonardo & Mondor, 2006; and modification of survival-reproduction tradeoffs: Emelianoff et al., 2008), or instruction of embryonic and tissue development (seen for example in nematodes: Landmann et al., 2014; cephalopods: McFall-Ngai, 2014; fish: Rawls et al., 2004; and mammals: Hooper & Gordon, 2001; Stappenbeck et al. 2002). Examples such as these serve to illustrate the critical interdependency between microbes and their hosts for regulating processes as fundamental as early tissue differentiation and metamorphosis. But the fact that this interdependency is not strictly limited to early development and instead persists through all stages and aspects of life (as reviewed in McFall-Ngai et al., 2013) naturally motivates the hypothesis that these immensely influential partnerships may

impact not just one host's life, but the evolutionary trajectories of entire host populations. And even though appreciation for the vital importance of these microbial symbioses for influencing host fitness has been increasing broadly (as reviewed in Gilbert et al., 2012), the role of host-symbiont interactions as a potential structuring feature of adaptive evolution remains poorly understood (Schluter, 2000; Futuyma, 2003; Janson et al., 2008).

What limited research we do have in this area suggests that the role microbial symbionts play in host diversification may indeed be significant: the dramatic radiations of Attini fungus-farming ants (Schultz & Brady, 2008; Ješovnik et al., 2016), *Asteromyia* gall midges (Stireman et al., 2010; Heath & Stireman, 2010, Joy, 2013), and Cicadellidae leafhoppers (Takiya et al., 2006; Moran et al., 2008) for example all illustrate that symbionts can influence the ecological radiation of their hosts (but see Bennett & Moran, 2015 for potential evolutionary constraints). Together, examples such as these highlight that the microbes residing in and on nearly all organisms 1) significantly influence all aspects of their hosts' lives, and 2) that the synergy between hosts and their microbiota makes it difficult to attribute ultimate fitness outcomes to either partner alone. These conclusions have helped motivate the proposal that adaptive radiations may be best understood not as the result of external environmental forces acting on a single organism, but rather as the result of these forces acting on *teams* of hosts and their associated microbiota (Sudakaran et al., 2017). But empirical studies addressing this novel hypothesis remain rare and much more work is needed to assess the validity of this perspective. In my dissertation I aimed to address this shortcoming through a first investigation of the putative role of the gut microbiota in the diversification of dung beetles in the genus *Onthophagus*.

ONTHOPHAGUS DUNG BEETLES

With over 2,300 extant species found in a broad variety of habitats, and on every continent except Antarctica, *Onthophagus* ranks among the most species-rich genera in the animal kingdom (Tarasov & Solodovnikov, 2011). This diversity is in part attributed to specialization on different dung types (Davis and Sutton, 1997; Emlen et al., 2005), as extant *Onthophagus* have radiated onto a remarkably diverse array of dung types (e.g., ungulates: Emlen et al., 2005; arboreal monkeys: Estrada et al., 1999; kangaroos and wallabies: Matthews, 1971). However, the exact same type of dung is often utilized by multiple *Onthophagus* species (Emlen et al., 2005), and especially in Europe several species complexes exist (Pizzo et al., 2006; Angus, 2008; Macagno et al., 2011) comprised of closely related and morphologically highly similar yet non-hybridizing species, suggesting that specialization on discrete dung types cannot be the sole ecological mechanism underlying *Onthophagus* diversity. At the same time, all dung types constitute a nutritionally incomplete and therefore challenging food source (Holter, 2016; Frank et al., 2017). It has long been hypothesized that dung beetles are able to process this diet through association with nutritional symbionts (Goidanich & Malan, 1962; Rougon et al., 1990), and recent work is beginning to support this hypothesis. Research now shows that *Onthophagus* beetles appear to utilize their food source through the association with symbionts that are vertically transmitted by mothers through the *pedestal*, a maternal fecal pellet onto which an egg is oviposited and which is consumed by larvae immediately after they hatch from the egg, and that animals deprived of their pedestal microbiota have reduced fitness (Estes et al., 2013; Shukla et al., 2013; Schwab et al., 2016). Outside dung beetles, such vertical transmission of microbes has the demonstrated potential to facilitate both host-symbiont coevolution, and crucially, also

opens up additional paths for host evolution through extra-genetic inheritance of microbial partners (reviewed in Shapira, 2016). The crucial contribution of vertically-transmitted symbionts to normal development on one side, and the remarkable diversity seen among ecologically similar *Onthophagus* species on the other, therefore raises the possibility that the radiation of *Onthophagus* beetles has been at least partially shaped by tight associations with vertically inherited, host-specific microbial communities. This dissertation seeks to investigate this hypothesis in a stepwise manner.

OUTLINE

In the second chapter of this dissertation, I sought to directly test the hypothesis of whether different *Onthophagus* beetle species have evolved to interact with, and benefit from, a host species-*specific* microbial community. To test this, I exchanged the maternally-provisioned, pedestal microbiota between two ecologically similar dung beetle species via reciprocal pedestal transplants. I hypothesized that beetles receiving a mismatched pedestal should have lower fitness compared to individuals provided with their own pedestal. This chapter has been published in *Ecological Entomology* (Parker et al., 2019). Then, in my third chapter, I sought to narrow the scope of my investigation of host-specific microbial communities and investigate the ecological and evolutionary conditions which create such differences. Specifically, I exchanged pedestal microbiota between two sympatric and syntopic sister species with the hypothesis that host-microbiota species specificity could arise over evolutionarily short timespans, including between recently diverged, broadly sympatric and often syntopic sister species who share virtually identical ecologies. This chapter has been submitted for publication and is currently in

review at *Ecological Entomology*. Next, in my fourth chapter I narrowed my focus further by investigating the role of the microbiome in the rapid range expansion of a single, exotic species across the Eastern United States. That is, I leveraged the ongoing range expansion of invasive *Onthophagus taurus* beetles from their initial introduction location in Northern Florida to the current invasion edge in Northern Michigan. I sought to test the hypothesis that vertically transmitted microbiota facilitate local thermal adaptations needed to survive across this cline, and therefore could be transferred across populations via microbiome transplantations. This chapter is published in *Ecology & Evolution* (Parker & Moczek 2020). Finally, my fifth chapter focuses on the taxonomic composition of the *Onthophagus* microbiome and how this composition is affected by introduction events. Specifically, I used a 16S sequencing approach to investigate which bacterial taxa are present in the microbiome of both native and exotic *O. taurus* populations – and how these microbial communities compare to those of other native *Onthophagus* species collected in these same locations. This chapter is published in *Microbial Ecology* (Parker et al. 2020). By using a variety of approaches and integrating across a number of levels of biological organization, my dissertation explores the role of the microbiome in host adaptation and diversification in a robust manner. Though my work was done in only one study system, it is my hope that my research is of broad interest and helps expand our understanding of the evolutionary and ecological implications of host-symbiont interactions generally.

REFERENCES

Angus, R. B. (2008) A chromosomal analysis of the *Onthophagus similis-opacicollis-fracticornis* species group (Coleoptera: Scarabaeidae). *Tijdschrift voor Entomologie*, 151(2), 235-244.

Bennett, G. M., and Moran, N. A. (2015) Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *PNAS*, 112(33), 10169-10176.

Davis, A. J., and Sutton, S. L. (1997) A dung beetle that feeds on fig: implications for the measurement of species rarity. *Journal of Tropical Ecology*, 13(5), 759-766.

Emelianoff, V., Chapuis, E., le Brun, N., Chiral, M., Moulia, C., and Ferdy, J. B. (2008) A Survival-Reproduction Trade-Off in Entomopathogenic Nematodes Mediated by Their Bacterial Symbionts. *Evolution*, 932-942.

Emlen, D. J., Marangelo, J., Ball, B., and Cunningham, C. W. (2005) Diversity in the Weapons of Sexual Selection: Horn Evolution in the Beetle Genus *Onthophagus* (coleoptera: Scarabaeidae). *Evolution*, 59(5), 1060–1084.

Estes, A. M., Hearn, D. J., Snell-Rood, E. C., Feindler, M., Feeser, K., Abebe, T., … Moczek, A. P. (2013). Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS ONE*, 8(11), 1–15.

Estrada, A., Anzures, D., and Coates-Estrada, R. (1999). Tropical rain forest fragmentation, howler monkeys (*Alouatta palliata*), and dung beetles at Los Tuxtlas, Mexico. *American journal of primatology*, 48(4), 253-262.

Frank, K., Brückner, A., Hilpert, A., Heethoff, M., & Blüthgen, N. (2017). Nutrient quality of vertebrate dung as a diet for dung beetles. *Scientific Reports*, 7(1), 1–12.

Futuyma, D. J. (2003) Accounting for biological diversity. *Evolution*, 57(5), 1216–1220.

Gilbert, S. F., Sapp, J., and Tauber, A. I. (2012) A symbiotic view of life: we have never been individuals. *The Quarterly review of biology*, 87(4), 325-341.

Goidanich, A., & Malan, C. E. (1962). Sulla fonte di alimentazione e sulla microflora aerobica del nido pedotrofico e dell'apparato digerente delle larve di scarabei coprogagi: (Coleoptera:scarabaeidae).

Hadfield, M. G. (2011) Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. *Annual Review of Marine Science* 3:453–70.

Heath, J. J., and Stireman, J. O. (2010) Dissecting the association between a gall midge, *Asteromyia carbonifera*, and its symbiotic fungus, *Botryosphaeria dothidea*. *Entomologia Experimentalis et Applicata*, 137(1), 36-49.

Holter, P. (2016). Herbivore dung as food for dung beetles: elementary coprology for entomologists. *Ecological Entomology*, 41(4), 367–377.

Hooper, L. V., and Gordon, J. I. (2001) Commensal host-bacterial relationships in the gut. *Science*, 292(5519), 1115-1118.

Janson, E. M., Stireman, J. O., Singer, M. S., and Abbot, P. (2008) Phytophagous insect–microbe mutualisms and adaptive evolutionary diversification. *Evolution*, 62(5), 997-1012.

Ješovnik, A., González, V. L., and Schultz, T. R. (2016) Phylogenomics and divergence dating of fungus-farming ants (Hymenoptera: Formicidae) of the genera *Sericomyrmex* and *Apterostigma*. *PloS one*, 11(7), e0151059.

Joy, J. B. (2013). Symbiosis catalyses niche expansion and diversification. *Proceedings of the Royal Society of London B: Biological Sciences*, 280(1756), 20122820.

Landmann, F., Foster, J. M., Michalski, M. L., Slatko, B. E., and Sullivan, W. (2014) Co-evolution between an endosymbiont and its nematode host: Wolbachia asymmetric posterior localization and AP polarity establishment. *PLoS neglected tropical diseases*, 8(8), e3096.

Leonardo, T. E., and Mondor, E. B. (2006) Symbiont modifies host life-history traits that affect gene flow. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1590), 1079-1084.

Macagno, A. L., Pizzo, A., Rolando, A., and Palestrini, C. (2011). Size and shape interspecific divergence patterns partly reflect phylogeny in an Onthophagus species-complex (Coleoptera: Scarabaeidae). *Zoological Journal of the Linnean Society*, 162(3), 482-498.

Matthews, E. G. (1971). A revision of the scarabaeine dung beetles of Australia. I. Tribe Onthophagini. *Australian Journal of Zoology Supplementary Series*, 19(9), 3-330.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V, Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229–3236.

McFall-Ngai, M. J. (2014) The Importance of Microbes in Animal Development: Lessons from the Squid-Vibrio Symbiosis. *Annual Review of Microbiology*, 68(1), 177–194.

Moczek, A. P. (2015). Re-evaluating the environment in developmental evolution. *Frontiers in Ecology and Evolution*, *3*, 7.

Moran, N. A., McCutcheon, J. P., and Nakabachi, A. (2008) Genomics and evolution of heritable bacterial symbionts. *Annual review of genetics*, 42, 165-190.

Parker, E. S., Dury, G. J., & Moczek, A. P. (2019). Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles. *Ecological Entomology*, *44*(2), 274-282.

Parker, E. S., & Moczek, A. P. (2020). Don't stand so close to me: Microbiota-facilitated enemy release dynamics in introduced *Onthophagus taurus* dung beetles. *Ecology and Evolution*.

Parker, E. S., Newton, I. L., & Moczek, A. P. (2020). (My Microbiome) Would Walk 10,000 miles: Maintenance and Turnover of Microbial Communities in Introduced Dung Beetles. *Microbial Ecology*, 1-12.

Pizzo, A., Roggero, A., Palestrini, C., Cervella, P., Del Pero, M., and Rolando, A. (2006) Genetic and morphological differentiation patterns between sister species: the case of *Onthophagus taurus* and *Onthophagus illyricus* (Coleoptera, Scarabaeidae). *Biological Journal of the Linnean Society*, 89(2), 197-211.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS*, 101(13), 4596-4601.

Rougon, D., Rougon, C., Levieux, J., & Trichet, J. (1990). Variations in the amino-acid content in zebu dung in the Sahel during nesting by dung-beetles (Coleoptera, Scarabaeidae). *Soil Biology and Biochemistry*, 22(2), 217–223.

Schluter, D. (2000) The ecology of adaptive radiation. *OUP Oxford*.

Schultz, T. R., and Brady, S. G. (2008) Major evolutionary transitions in ant agriculture. *PNAS*, 105(14), 5435-5440.

Schwab, D. B., Riggs, H. E., Newton, I. L. G., & Moczek, A. P. (2016). Developmental and Ecological Benefits of the Maternally Transmitted Microbiota in a Dung Beetle. *The American Naturalist*, 188(6), 000–000.

Shapira, M. (2016) Gut microbiotas and host evolution: scaling up symbiosis. *Trends in ecology and evolution*, 31(7), 539-549.

Shikuma, N. J., Pilhofer, M., Weiss, G. L., Hadfield, M. G., Jensen, G. J., & Newman, D. K. (2014). Marine Tubeworm Metamorphosis Induced by Arrays of Bacterial. *Science*, 343(January), 529–534.

Shukla, S. P., Sanders, J. G., Byrne, M. J., & Pierce, N. E. (2016). Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular Ecology*, *25*(24), 6092–6106.

Stappenbeck, T. S., Hooper, L. V., and Gordon, J. I. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *PNAS*, 99(24), 15451-15455.

Stireman III, J. O., Devlin, H., Carr, T. G., and Abbot, P. (2010) Evolutionary diversification of the gall midge genus *Asteromyia* (Cecidomyiidae) in a multitrophic ecological context. *Molecular Phylogenetics and Evolution*, 54(1), 194-210.

Sudakaran, S., Kost, C., & Kaltenpoth, M. (2017). Symbiont acquisition and replacement as a source of ecological innovation. *Trends in Microbiology*, *25*(5), 375-390.

Takiya, D. M., Tran, P. L., Dietrich, C. H., and Moran, N. A. (2006) Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Molecular Ecology*, 15(13), 4175-4191.

Tarasov, S. I., & Solodovnikov, A. Y. (2011). Phylogenetic analyses reveal reliable morphological markers to classify mega-diversity in Onthophagini dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae). *Cladistics*, 27(5), 490-528.

CHAPTER 2

Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles

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ABSTRACT

1. The significance of host-microbe interactions is increasingly appreciated across biological disciplines, yet to what extent these interactions influence developmental outcomes within and across generations remains poorly understood.
2. We investigated the putative role of host-microbe interactions in the adaptive diversification of *Onthophagus* dung beetles, one of the most species-rich and ecologically successful genera of insects; *Onthophagus* mothers vertically transmit growth and fitness enhancing gut symbionts to their offspring through a fecal secretion known as the pedestal.
3. We reciprocally exchanged pedestals between two ecologically similar congeneric *Onthophagus* species to assess the degree to which pedestal microbiota from one species can substitute for those of another.

4. We find that the presence of a heterospecific pedestal delays development and increases mortality, and that the fitness costs of non-host-specific microbiota are maintained transgenerationally.
5. Collectively, our results support the hypothesis that *Onthophagus* beetles maintain, interact with, and are dependent upon host species-specific microbial communities to support normal growth and development. We discuss the implications of our results in the context of host microbiota co-evolution.

INTRODUCTION

Understanding the processes that enable and shape the generation of biodiversity is a major objective of evolutionary biology. Traditionally, these processes have been explained as the result of divergent selective pressures acting on generations of individuals. However, increasing appreciation for the vital role of host-associated microbial symbionts has added complexity to this notion of individuality, as microbial associates have been shown to exert a diverse array of influences on their animal hosts, including nutritional supplementation (reviewed in Douglas, 2009), life history decisions (such as metamorphosis induction: Hadfield, 2011; Shikuma *et al.*, 2014; reproductive timing: Leonardo & Mondor, 2006; and modification of survival-reproduction tradeoffs: Emelianoff *et al.*, 2008), or instruction of embryonic and post-embryonic tissue development (seen for example in nematodes: Landmann *et al.*, 2014; cephalopods: McFall-Ngai, 2014; fish: Rawls *et al.*, 2004; and mammals: Hooper and Gordon, 2001; Stappenbeck *et al.* 2002).

Even though awareness and appreciation for the importance of host-microbiota interactions in structuring developmental outcomes has been increasing broadly (reviewed in

Gilbert *et al.*, 2012), the evolutionary consequences of disruptions to these interactions remains poorly understood. The importance of the microbiota for normal development especially in partnerships involving strict, vertical transmission of microbial symbionts (for example in aphids: Koga *et al.*, 2012; shield bugs: Salem *et al.*, 2015; and leafhoppers: Watanabe *et al.*, 2014), has raised the possibility that alteration or disruption of these communities may result in fitness consequences within and across generations, thereby affecting host evolution. Indeed, recent work suggests that gut microbiota disruptions in *Drosophila* lead to transgenerational alterations in development and mate preferences (Sharon *et al.*, 2010; Morimoto *et al*., 2017), however, some of these effects have been difficult to replicate (Leftwich *et al*., 2017). Overall, empirical studies addressing this hypothesis remain rare and more work is needed to determine the broader validity of this perspective. Here, we investigate the role of gut microbiota in influencing the development and potential fitness of dung beetles within and across generations. We focus on beetles in the genus *Onthophagus*, one of the most dramatic radiations of terrestrial invertebrates, and assess the extent to which different *Onthophagus* host species have evolved to utilize host-specific microbial partners.

Onthophagus are considered true dung beetles in that they rely on dung throughout their life cycle. With over 2000 extant species (Tarasov & Kabakov, 2010) *Onthophagus* ranks among the most species-rich genera in the animal kingdom. This diversity is in part attributed to resource specialization (Davis and Sutton, 1997; Emlen *et al.*, 2005) as extant *Onthophagus* have radiated onto a remarkable diversity of dung types (e.g. ungulates: Emlen *et al.*, 2005; arboreal monkeys: Estrada *et al.*, 1999; kangaroos and wallabies: Matthews, 1971). However, the same type of dung is often utilized by multiple *Onthophagus* species (Emlen *et al.*, 2005), and especially in Europe several species complexes exist (Pizzo *et al.*, 2006; Angus, 2008; Macagno

et al., 2011) comprised of closely related and morphologically highly similar yet non-hybridizing species, suggesting that specialization on discrete dung types cannot be the sole ecological mechanism underlying *Onthophagus* diversity. At the same time, all dung types constitute challenging food sources that are generally low in nutritional value, lack essential amino acids, and are comprised mainly of hard to digest cellulose (Muller, 1980). Recent work suggests *Onthophagus* beetles utilize this food source through the association with symbionts that are vertically transmitted by mothers through the *pedestal*, a maternal fecal pellet onto which an egg is oviposited and which is consumed upon hatching of the larva (Estes *et al.*, 2013; Schwab *et al.*, 2016; Schwab *et al.*, 2017). Experimental removal of maternally transmitted symbionts in *Onthophagus* results in delayed development and reduced growth, as well as elevated mortality under stressful conditions, whereas re-inoculation with experimentally cultured pedestal microbiota restores normative development (Schwab *et al.*, 2016). The significance of vertically-transmitted symbionts to normal development in *Onthophagus*, coupled with the remarkable diversity seen among ecologically similar *Onthophagus* species, raises the possibility that the diversification of *Onthophagus* has been at least partially shaped by the developmental symbioses formed between beetle hosts and their symbionts. Here we assess an important prediction of this hypothesis.

Specifically, we aimed to examine whether dung beetle host species have evolved to interact with, and benefit from, a host-specific microbial community. To test this, we exchanged the maternally-provisioned microbiota between two ecologically similar, congeneric, generalist feeding dung beetle species — *Onthophagus gazella* (Fabricius, 1787) and *O. sagittarius* (Fabricius, 1775) — derived from a location where both species occur in sympatry. We hypothesized that if the microbiota of each species provides functionally unique benefits to its

host, beetles receiving a mismatched (i.e. heterospecific) microbiota should have compromised growth, as well as developmental and survival outcomes compared to individuals provided their own, host-species specific microbiota. We further predicted that if microbiota-host mismatches cause adverse fitness consequences, these effects should be transgenerational, as at least a subset of the mismatched microbes would be vertically transmitted to the next generation.

MATERIALS AND METHODS

Beetle collection and husbandry

Onthophagus gazella and *O. sagittarius* beetles were field collected in Gatton and Imbil near Brisbane, Queensland, Australia, and shipped to Bloomington, IN. *Onthophagus sagittarius* and *O. gazella* are native to southeast Asia and much of Africa, respectively, and in their native ranges do not occur in sympatry. However, both species have been introduced into Australia approximately 50 years ago as part of a biocontrol effort aimed to enhance the management of dung and dung breeding flies (Edwards, 2007). Both species are now well established and broadly overlapping throughout their exotic range.

Colonies of both species were maintained in a sand/soil mixture at 28°C and fed antibiotic-free cow manure twice a week as described in Moczek (2006). Experimental animals were generated by breeding six females and three males in a small sand/soil filled container with *ad libitum* food. Following protocols established in Schwab *et al.* (2016, 2017), brood balls produced by adult females were collected after five days and carefully opened by hand. Eggs were extracted using autoclave sterilized paintbrushes. After removal, eggs were then surface sterilized by 100µL of 1% bleach (final concentration of 0.0525% sodium hypochlorite) and 0.1% Triton-X 100 solution followed by two rinses of 1mL of deionized water. Concurrently, the

interior surface upon which the egg had been positioned by the mother (referred to as the *pedestal*) was extracted using a flame sterilized surgical blade following Schwab *et al.* (2016). The pedestal was then placed at the center of an artificial brood ball within the well of a twelve-well plate prepared as described in Shafiei *et al.* (2001) and Schwab *et al.* (2016). A sterile paintbrush was then used to place a single sterile egg on the dissected pedestal, after which the artificial brood ball containing egg and pedestal were gently covered with dung to prevent desiccation. Eggs obtained from each species were haphazardly assigned to one of two treatments: a self-inoculated treatment wherein each surface sterilized egg was placed on its own pedestal, or a cross-inoculated treatment wherein each surface sterilized egg was placed on a pedestal obtained from the other species, resulting in two treatment groups per species, and a total of four treatments. For logistical reasons we could only accommodate two treatment groups, and chose the control group which would permit the most extreme contrast between microbiota origins (different species versus entirely self). A maximum of 3 individuals per treatment were placed in rows at random locations within each of the twelve-well plates used.

Twelve-well plates were then transferred to a 28°C incubator and checked once every 48 hours to assess larval progression in development as described below. The orientation and position of plates within the incubator were changed every 48 hours to minimize the effects of any potential microclimatic variation within the incubator.

Assessing the effects of microbiota swapping on growth, development and survival

To assess potential effects of microbiota swapping on growth, developmental rate, and survival we collected the following measurements for each individual: time, in days, to i) final (third) larval instar, ii) pupation, and iii) adulthood. We measured larval peak mass operationally

defined as the larval mass 48h after a given individual was first scored as a third instar (i.e. approximately day 3 or 4 of the final instar, or roughly 12 days since egg treatment for a self-inoculated animal), as well as pupal mass. All mass measurements were obtained to the nearest 0.0001g using a Mettler Toledo AL54 (Mettler, Columbus, Ohio, USA) scientific scale. Animals were sexed as pupae to enable the analysis of sex-bias. Lastly, we measured survival to adulthood as well as adult body size. Adult size was measured as thorax (pronotum) width using a two-dimensional morphometric setup consisting of a Leica dissecting microscope, a Scion digital camera and ImageJ v1.44p software as previously described (Moczek, 2006).

Assessing transgenerational effects of microbiota manipulations

To assess whether microbiota manipulations affected fitness beyond the initial generation of larvae, we used surviving F_1 adults to rear a second generation of beetles. However, because not a single *O. sagittarius* female receiving a *O. gazella* pedestal survived to reach adult sexual maturity (out of a total of 23; see below), this experiment was restricted to *O. gazella* only.

To obtain second generation individuals, virgin adult F_1 *O. gazella* females were maintained in two female-only, treatment-specific colonies following the protocol described above for at least three weeks to allow for sexual maturation. Then, male *O. gazella* from the original field collected colony were added to each female treatment-specific colony to generate an approximately 2:1 female to male ratio. Male and female *O. gazella* were maintained together for six days, after which females were separated and placed individually in single-female breeding containers and provided *ad libitum* dung. After five days, brood balls containing F_2 offspring were collected. Each brood ball was weighed and then uniquely labeled and stored in an

individual, small soil/sand filled container until adult emergence. Emerging F_2 beetles were sexed and then measured for body size as described above.

Statistical analyses

The effects of microbiota swapping on F_1 beetle development and fitness was assessed using a Welch's t-test to compare means of the two treatment groups for *O. gazella*. Differences in survival to adulthood between treatments was assessed using Pearson's chi-square tests.

To investigate the specific influence of the transgenerational effect of microbiota swapping on F_2 beetle development and fitness, we constructed linear and generalized linear (binomial family, logit link) models regressing F_2 adult body size, developmental time, and survival on all possible main effect combinations of maternal size, brood ball weight, sex, and microbiota identity. In each of these models, we tested for a possible interaction between maternal size and brood ball weight as previous work has shown a correlation between these two measures (Hunt & Simmons, 2002; Macagno *et al.*, 2018). The regressors included in each model were validated using F-tests, and regression diagnostics were performed to assess assumptions related to the constancy of variance, normality of the residuals, and to identify any outlying or otherwise particularly influential single points. All analyses were executed in R using RStudio and the *car*, *realimpo*, and base packages (Fox *et al.*, 2012; Grömping, 2013; RCore Team, 2013; RStudio Team, 2015). All figures were generated in RStudio using the *ggplot2*, *gridextra*, *ggsignif*, and *visreg* packages (Auguie, 2016; Wickham, 2016; Ahlmann-Eltze, 2017; Breheny & Burchett, 2017).

RESULTS

Microbiota swapping prolongs development time, reduces growth, and affects survival in both Onthophagus *species*

We sought to determine whether the vertically transmitted microbiota of *O. gazella* and *O. sagittarius* provide benefits specific to each host species. To do so we performed reciprocal swaps of the maternally provisioned pedestal, i.e. the main conduit through which mothers bequeath maternal microbiota unto their larval offspring (Schwab *et al.* 2016). We then measured several metrics related to growth, development, and survival among treatment groups. We predicted that if *Onthophagus* microbiota provide host species-specific benefits, this should manifest in prolonged development, reduced maximal growth, or decreased survival among cross-inoculated compared to self-inoculated individuals. Our results provide partial support for these predictions.

Specifically, *O. gazella* individuals that received an *O. sagittarius* pedestal took significantly longer to reach both pupation and adulthood yet weighed significantly less as pupae than *O. gazella* eggs which were provided their original pedestal (Welch's t-test: $t_{\text{pupation}} = 3.6965$, $P_{\text{pupation}} = 0.0012$; $t_{\text{adulthood}} = 2.9875$, $P_{adulthood} = 0.0076$; $t_{\text{weight}} = 2.373$, $P_{\text{weight}} = 0.0288$; fig. 1). None of these treatment effects differed significantly among sexes (Welch's t-test: $t_{\text{pupation}} = 1.3532$, $P_{\text{pupation}} = 0.1834$; $t_{\text{adulthood}} = 1.0755$, $P_{adulthood} = 0.2886$; $t_{\text{weight}} = 0.1895$, $P_{\text{weight}} = 0.8506$). Conversely, we detected no significant treatment effect with respect to peak larval weight ($P = 0.2807$), time needed to reach the third larval instar ($P = 0.3185$), nor adult body size ($P = 0.1371$). Further, even though survival to adulthood was lower in cross-inoculated individuals (48%) compared to self-inoculated individuals (71%) this effect was not statistically significant (X^2 test: $X^2 = 1.7145$, $P = 0.1904$; fig. 2).

In contrast, we recovered a significant effect of pedestal origin on survival to adulthood in *O. sagittarius*: when reared on their original *O. sagittarius* pedestal, 16/32 (50%) individuals survived to pupa and 14 (44%) to adulthood, respectively. However, when reared on an *O. gazella* pedestal, only 5/23 (22%) individuals survived to the final larval instar, of which only 3 individuals (two males, one female; 13%) reached the pupal and subsequent adult stages (X^2 test: $X^2 = 4.5580$, $P = 0.0328$; fig. 2). Unfortunately, this high mortality in the cross-inoculated treatment group severely reduced sample sizes for comparisons of growth and developmental time metrics, precluding meaningful statistical inference for this species.

Negative effects of microbiota swapping are transgenerational

Lastly, we sought to investigate whether any developmental or fitness effects due to heterospecific microbiota inoculation would recur in subsequent generations, as would be predicted if functionally relevant microbes are vertically inherited. Specifically, we tested the prediction that offspring of mothers who as larvae were forced to develop utilizing a heterospecific pedestal would exhibit reduced growth performance and survival compared to offspring from mothers who as larvae had access to their species-specific pedestal. Further, if such an effect existed we sought to compare it to other factors identified by previous work to influence adult body size, in particular maternal size and brood ball weight (Hunt & Simmons, 2002). Due to the high mortality seen in one of the two *O. sagittarius* treatments we were only able to execute this experiment in *O. gazella*.

In contrast to earlier studies (e.g. Macagno *et al.*, 2018) we did not detect a significant effect of brood ball weight, maternal size, or their interaction when added to models already containing offspring sex and microbiota origin. Furthermore, the two variables were not

correlated (Pearson's correlation coefficient: r = -0.03). However, we did find a significant effect of offspring sex (one-way ANOVA: F = 80.5198, P < 0.0001), and to a slightly lesser magnitude, microbiota origin (one-way ANOVA: F = 21.6109, P < 0.0001), on adult F_2 offspring size. That is, after controlling for the effects of offspring sex, offspring of *O. gazella* larvae inoculated with their species-specific microbiota (n = 39) developed to significantly larger adult sizes compared to offspring of parents inoculated with microbiota derived from *O. sagittarius* (n = 34; fig.3). In contrast, no such effect was seen in survival rate or development time to adulthood. These results suggest that at least some of the developmental consequences introduced through microbiota swapping persist across generations.

DISCUSSION

A large body of work demonstrates the diverse benefits that hosts derive from their microbial partners (as reviewed in McFall-Ngai *et al.*, 2013). These findings have motivated the hypothesis that host development may in part be shaped by the symbiotic partnerships hosts form, and that evolutionary outcomes may in part be influenced by these interactions (Gilbert *et al.*, 2015). However, the role and significance of host microbiota influences on long-term evolutionary trajectories of their hosts remain poorly understood. Here we investigate the possible role of the pedestal microbiota in the diversification of dung beetles in the genus *Onthophagus*, one of the most dramatic radiations of insects, by assessing the extent to which *Onthophagus* host species have evolved to utilize specific microbial partners. We find i) that individuals provisioned with the microbiota of a congeneric beetle host exhibit lower fitness compared to individuals raised on their species-specific microbiota, and ii) that a subset of fitness reductions seen in cross-

inoculated individuals are maintained transgenerationally. Below we discuss the most important implications of our results.

Inoculation with non-host specific microbiota reduces fitness

We find that individuals inoculated with microbiota derived from a heterospecific host exhibit significantly reduced growth and prolonged development time (*Onthophagus gazella*), or elevated mortality (*O. sagittarius*). These results support the hypothesis that these two relatively distantly related *Onthophagus* species, which diverged roughly 37 million years ago (see Emlen *et al.*, 2005 fig. 3; Breeschoten *et al.*, 2016), are adapted to utilize host specific microbial communities (fig. 1 and 2). Importantly, these results were obtained from populations collected from a relatively recently established range in which both species have been occurring in sympatry and frequently in syntopy, i.e. the same dung pads, for approximately 50 years, or roughly one hundred generations. These observations suggest that even though these species now occur in extremely close proximity, both appear to have maintained disparate, and functionally non-equivalent microbiota. However, it is worth noting that our choice of a control group – eggs returned to their own, original pedestal - does not allow us to separate host species-specific and maternal line-specific contribution to the role of the microbiota in determining offspring fitness and development. To address this issue future work should specifically contrast the developmental outcomes of individuals provided microbiota of strictly maternal origin to those receiving microbiota from unrelated adults.

Furthermore, we found that the magnitude of fitness reductions seen in the F_1 generation was not fully equivalent in both species. Cross-inoculated *O. sagittarius* beetles survived at an extremely low rate (only 13% reached adulthood) when compared to both the self-inoculated

treatment and the cross-inoculated *O. gazella* beetles. Though this low survival rate precluded statistical comparisons of other developmental metrics between the two *O. sagittarius* treatment groups, the difference in survival rates between the two species suggests that *O. sagittarius* beetles may generally be more sensitive to microbiota disruptions. It is unclear at this point whether this reduced survival is due to the loss of beneficial microbes, or the introduction of microbes which become pathogenic in a new host. To clarify this, future work is needed to characterize i) the composition of the undisrupted gut microbial communities of these two beetle species, ii) how the compositions of these communities are altered through pedestal transplanting, and iii) if transplanting allows latent pathogenic bacteria to overwhelm their new hosts. Regardless of this current ambiguity, these results support the hypothesis that ecologically similar *Onthophagus* beetles have evolved to obtain host-specific benefits from diverse and functionally non-equivalent microbial communities.

Fitness costs due to non-host-specific microbiota are transgenerational

Previous work has shown that in a third species, *Onthophagus taurus* (Schreber, 1759), mothers vertically transmit gut microbes to their offspring through the pedestal (Estes *et al.*, 2013). We reasoned therefore that negative fitness effects due to host-microbiota mismatching seen in the F_1 generation of *O. sagittarius* and *O. gazella* should be at least partially maintained in subsequent generations, as cross-inoculated larvae would, as adults, pass on the non-host specific microbiota through their own pedestal. In support of our prediction, we found that the offspring of cross-inoculated *O. gazella* mothers were significantly smaller as adults than the offspring of self-inoculated *O. gazella* mothers. The effect of microbiota origin on F_2 size remained significant in a model that also accounted for the effects of sex, brood ball size, and maternal size (fig. 3). This

transgenerational effect of pedestal swapping provides additional support for the hypothesis that vertical transmission of host specific microbiota is a common feature of *Onthophagus* beetles. Moreover, it provides further support for the prediction that the benefits provided by these vertically inherited communities may be the result of a history of coevolution.

Surprisingly, F_2 males were found to be significantly larger than F_2 females (fig. 3), yet no such effect was seen in the F_1 generation. To our knowledge, this is the first demonstration of such a pronounced sex-biased body size dimorphism in *Onthophagus* generally, and *O. gazella* specifically, though the mechanism underlying this result is unclear. One potential explanation could be that pre-adult mortality in our study primarily affected small males, yet sex ratios of survivors were even (36 males vs. 39 females) and the mass distribution of the brood balls of individuals who did not reach adulthood, a measure which is generally tightly correlated with both offspring body size and male mortality rates (Hunt & Simmons, 2002; House *et al*., 2011), was not significantly different from those of the survivors, failing to support this hypothesis.

Transgenerational effects of gut microbes in insects have also been uncovered by other studies. Recent work in *Drosophila melanogaster* (Meigen, 1830), for example, shows that daughters of parents inoculated with *Acetobacter pomorum* are significantly smaller than daughters of parents inoculated with other bacterial strains (Morimoto *et al.*, 2017). Furthermore, body size in *D. melanogaster* is positively associated with numerous fitness metrics such as fecundity, fertilization success, and attractiveness — all of which are related to the strength of sexual selection in populations (Bonduriansky, 2001; Byrne & Rice, 2006; Morimoto *et al.*, 2016). Similarly, our work shows that both daughters and sons of cross-inoculated parents are significantly smaller as adults than offspring of self-inoculated parents. Like in *D. melanogaster*, body size in *Onthophagus* beetles is positively associated with fecundity, survival, and offspring

quality in females (Hunt & Simmons, 2000; Hunt & Simmons, 2002), and fighting success in males (Moczek & Emlen, 2000). Thus, our findings lend additional support to the hypothesis that the maintenance of host specific gut microbiota associations may be critical for many life history traits, and conversely, that disruptions of these associations may affect population health and persistence. Future studies should aim to directly assess the impact of gut microbiota disruptions on *Onthophagus* population dynamics over time.

Diverse ecological factors may structure host specific microbiota in Onthophagus

Dung constitutes a challenging diet for insects given the abundance of macromolecules that are hard to digest, such as cellulose and lignin, and the simultaneous absence of key nutrients such as essential amino acids (Muller, 1980). It is therefore conceivable that dung beetle hosts utilize specific microbiota in order to meet the challenges imposed by such a diet, and that over time such partnerships result in interactions and dependencies specific to a given host beetle species and microbiota. Another role for the microbiota in *Onthophagus* may be the production of antimicrobial compounds as *Onthophagus* beetles develop in close proximity to diverse fungi, including entomophagic taxa such as *Metarhizium* (Chouvenc *et al.*, 2013; Estes *et al.*, 2013; Rosengaus *et al.*, 2013; Shukla *et al.*, 2016). Recent work shows that experimental inoculation with maternal microbiota significantly reduces mortality in *Onthophagus* dung beetles following standardized exposure to *Metarhizium* spores compared to inoculation with random soil microbes or PBS alone (Schwab *et al.*, in preparation). This raises the possibility that host species may also associate with microbiota to aid in defense against fungal attacks, providing additional pressure to maintain associations between beetle hosts and their microbial partners.

Other research has suggested that most species acquire their microbiota not through strict vertical or horizontal transmission, but through some combination of the two (Shapira, 2016; Moran & Sloan, 2015). Our findings suggest that this is likely also the case for the two dung beetle species examined here. Cross-inoculated F_1 *O. gazella* beetles survived to adulthood at higher rates than cross-inoculated *O. sagittarius* beetles, but in neither case did the loss of these specific microbial communities lead to the complete mortality of a host line. This result suggests both species were able to assemble functionally compensatory microbial communities in the absence of their vertically transmitted microbiota, though *O. sagittarius* appeared less able to do so for reasons currently unclear to us. We speculate this may be due to a greater reliance on strict vertical transmission of the microbiota in this species. The observation of compensation in the absence of normally vertically transmitted microbiota is not surprising; even extremely tight host-symbiont associations such as between aphids and *Buchnera aphidicola* can break down and be successfully replaced by newly acquired symbionts (Chong & Moran, 2018). Future research could leverage the *Onthophagus* system to explore the drivers of microbial community assembly by examining the extent to which different *Onthophagus* species rely on vertical or horizontal transmission, or varying combinations thereof, and under what conditions transmission routes may change.

Onthophagus *beetles as a model system for the study of the evolutionary ecology of symbiosis*

The hologenome theory of evolution suggests that selection acting on a host and its associated microbiota together (a holobiont) should lead to phylosymbiosis, or a microbiota community assembly concordant with host phylogenetic distance (Zilber-Rosenberg, 2008; Theis et. al., 2016). This in turn predicts that increasing host phylogenetic distance should correlate with

increasing levels of host-microbiota incompatibility (Brooks *et al.*, 2016). In this study we have shown that two *Onthophagus* species whose last common ancestor existed approximately 37 million years ago exhibit measurable adverse fitness and developmental effects when subjected to cross-inoculation. Future work could leverage the number of experimentally accessible species across both greater and narrower phylogenetic distances (Emlen *et al.*, 2005) to test this prediction in a comparative fashion across multiple clade members.

At the same time, the history of accidental and deliberate introductions seen in numerous *Onthophagus* species, coupled with the experimental tractability of this genus, offers exciting opportunities to test if and how hosts, their microbiota, and the interactions between them evolve in novel environments (Edwards, 2007; Silva et al., 2016). Studies examining the role of host-symbiont interactions in potentially structuring range expansions into novel, challenging, habitats are particularly well suited for study within this system. Current work is investigating if and how *Onthophagus* gut microbiota change when host populations invade novel environments, knowledge that will further our understanding of the potential role of gut microbiota in shaping the ecological radiation of *Onthophagus* species and populations.

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REFERENCES

Ahlmann-Eltze, C. (2017) ggsignif: Significance Brackets for 'ggplot2'. R package version 0.4.0.

Angus, R. B. (2008) A chromosomal analysis of the Onthophagus similis-opacicollis-fracticornis species group (Coleoptera: Scarabaeidae). Tijdschrift voor Entomologie, 151(2), 235-244.

Auguie, B. (2016) gridExtra: Miscellaneous Functions for "Grid" Graphics. R package version 2.2.1.

Bonduriansky, R. (2001). The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews*, 76(3), 305-339.

Breeschoten, T., Doorenweerd, C., Tarasov, S., and Vogler, A. P. (2016) Phylogenetics and biogeography of the dung beetle genus Onthophagus inferred from mitochondrial genomes. *Molecular phylogenetics and evolution*, 105, 86-95.

Breheny, P. and Burchett, W. (2017) Visualization of Regression Models Using visreg. The R Journal, 9: 56-71.

Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., and Bordenstein, S. R. (2016) Phylosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. *PLoS biology*, 14(11), e2000225.

Byrne, P. G., & Rice, W. R. (2006). Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1589), 917-922.

Chong, R. A., & Moran, N. A. (2018) Evolutionary loss and replacement of *Buchnera*, the obligate endosymbiont of aphids. *ISME Journal*, 12(3), 898–908.

Chouvenc, T., Efstathion, C. A., Elliott, M. L., and Su, N. Y. (2013) Extended disease resistance emerging from the faecal nest of a subterranean termite. *Proceedings of the Royal Society of London B: Biological Sciences,Proc. R. Soc. B*, 280(1770), 20131885.

Davis, A. J., and Sutton, S. L. (1997) A dung beetle that feeds on fig: implications for the measurement of species rarity. *Journal of Tropical Ecology*, 13(5), 759-766.

Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23(1), 38–47.

Edwards, P. (2007) Introduced Dung Beetles in Australia 1967-2007, 1–66.

Emelianoff, V., Chapuis, E., le Brun, N., Chiral, M., Moulia, C., and Ferdy, J. B. (2008) A Survival-Reproduction Trade-Off in Entomopathogenic Nematodes Mediated by Their Bacterial Symbionts. *Evolution*, 932-942.

Emlen, D. J., Marangelo, J., Ball, B., and Cunningham, C. W. (2005) Diversity in the Weapons of Sexual Selection: Horn Evolution in the Beetle Genus *Onthophagus* (coleoptera: Scarabaeidae). *Evolution*, 59(5), 1060–1084. 3820.2005.tb01044.x

Estes, A. M., Hearn, D. J., Snell-Rood, E. C., Feindler, M., Feeser, K., Abebe, T., et al. (2013) Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS ONE*, 8(11), 1–15.

Estrada, A., Anzures, D., and Coates-Estrada, R. (1999). Tropical rain forest fragmentation, howler monkeys (Alouatta palliata), and dung beetles at Los Tuxtlas, Mexico. *American journal of primatology*, 48(4), 253-262.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., et al. (2012) Package 'car'. Vienna: R Foundation for Statistical Computing.

Gilbert, S. F., Bosch, T. C. G., & Ledón-Rettig, C. (2015). Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. *Nature Reviews Genetics*, 16(10), 611–622.

Gilbert, S. F., Sapp, J., and Tauber, A. I. (2012) A symbiotic view of life: we have never been individuals. *The Quarterly review of biology*, 87(4), 325-341.

Grömping, U. (2006) Relative Importance for Linear Regression in R: The Package relaimpo. JSS Journal of Statistical Software, 17(1).

Grömping, U., and Matthias, L. (2013) Package 'relaimpo'.

Hadfield, M. G. (2011) Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. *Annual Review of Marine Science* 3:453–70.

Hooper, L. V., and Gordon, J. I. (2001) Commensal host-bacterial relationships in the gut. *Science*, 292(5519), 1115-1118.

Hunt, J., and Simmons, L. W. (2000). Maternal and paternal effects on offspring phenotype in the dung beetle Onthophagus taurus. *Evolution*, 54(3), 936-941.

Hunt, J., and Simmons, L. W. (2002) The genetics of maternal care: Direct and indirect genetic effects on phenotype in the dung beetle Onthophagus taurus. *Proceedings of the National Academy of Sciences*.

Koga, R., Meng, X. Y., Tsuchida, T., & Fukatsu, T. (2012). Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. *Proceedings of the National Academy of Sciences*, 109(20), E1230-E1237.

Landmann, F., Foster, J. M., Michalski, M. L., Slatko, B. E., and Sullivan, W. (2014) Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric

posterior localization and AP polarity establishment. *PLoS neglected tropical diseases*, 8(8), e3096.

Leftwich, P. T., Clarke, N. V. E., Hutchings, M. I., & Chapman, T. (2017). Gut microbiomes and reproductive isolation in Drosophila. *Proceedings of the National Academy of Sciences*.

Leonardo, T. E., and Mondor, E. B. (2006) Symbiont modifies host life-history traits that affect gene flow. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1590), 1079-1084.

Macagno, A. L., Pizzo, A., Rolando, A., and Palestrini, C. (2011). Size and shape interspecific divergence patterns partly reflect phylogeny in an *Onthophagus* species-complex (Coleoptera: Scarabaeidae). *Zoological Journal of the Linnean Society*, 162(3), 482-498.

Macagno, A. L., Zattara, E. E., Ezeakudo, O., Moczek, A. P., and Ledón-Rettig, C. C. (2018). Adaptive maternal behavioral plasticity and developmental programming mitigate the transgenerational effects of temperature in dung beetles. *Oikos*.

Matthews, E. G. (1971). A revision of the scarabaeine dung beetles of Australia. I. Tribe Onthophagini. *Australian Journal of Zoology Supplementary Series*, 19(9), 3-330.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, 110(9), 3229-3236.

McFall-Ngai, M. J. (2014) The Importance of Microbes in Animal Development: Lessons from the Squid-Vibrio Symbiosis. *Annual Review of Microbiology*, 68(1), 177–194.

Moczek, A. P., and Emlen, D. J. (2000). Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes?. *Animal behaviour*, 59(2), 459-466.

Moczek, A. P. (2006) Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *The American Naturalist*, 168(6), 711-729.

Moran, N. A., and Sloan, D. B. (2015) The Hologenome Concept: Helpful or Hollow? *PLoS Biology*, 13(12), 1–10.

Morimoto, J., Pizzari, T., Wigby S. (2016) Developmental environment effects on sexual selection in male and female Drosophila melanogaster. *PLoS ONE* 11, e0154468.

Morimoto, J., Simpson, S. J., & Ponton, F. (2017) Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, 13, 20160966.

Muller, Z. O. (1980) Feed from animal wastes: state of knowledge. *FAO Animal Production and Health Paper*, (18).

Pizzo, A., Roggero, A., Palestrini, C., Cervella, P., Del Pero, M., and Rolando, A. (2006) Genetic and morphological differentiation patterns between sister species: the case of *Onthophagus taurus* and *Onthophagus illyricus* (Coleoptera, Scarabaeidae). *Biological Journal of the Linnean Society*, 89(2), 197-211.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, 101(13), 4596-4601.

Rosengaus, R. B., Mead, K., Du Comb, W. S., Benson, R. W., and Godoy, V. G. (2013) Nest sanitation through defecation: antifungal properties of wood cockroach feces. *Naturwissenschaften*, 100(11), 1051-1059.

RCore Team (2013) R: A language and environment for statistical computing.

RStudio Team (2015) RStudio: integrated development for R. RStudio, Inc., Boston, MA

Salem, H., Florez, L., Gerardo, N., & Kaltenpoth, M. (2015). An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society of London B: Biological Sciences*, 282(1804), 20142957.

Shafiei, M., Moczek, A. P., and Nijhout, H. F. (2001) Food availability controls the onset of metamorphosis in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Physiological Entomology*, 26(2), 173-180.

Shapira, M. (2016) Gut microbiotas and host evolution: scaling up symbiosis. *Trends in ecology and evolution*, 31(7), 539-549.

Sharon, G., Segal, D., Ringo, J. M., Hefetx, A., Zilber-Rosenberg, I., & Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 110(12), 4852.

Shikuma, N. J., M. Pilhofer, G. L. Weiss, M. G. Hadfield, G. J. Jensen, and D. K. Newman. (2014) Marine tubeworm metamorphosis induced by arrays of bacterial phage tail-like structures. *Science* 343:529–533.

Silva, D. P., Vilela, B., Buzatto, B. A., Moczek, A. P., & Hortal, J. (2016). Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, 18(11), 3137–3148.

Stappenbeck, T. S., Hooper, L. V., and Gordon, J. I. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences*, 99(24), 15451-15455.

Tarasov, S. I., and Kabakov, O. N. (2010) Two new species of Onthophagus (Coleoptera: Scarabaeidae) from Indochina, with a discussion of some problems with the classification of *Serrophorus* and similar subgenera. *Zootaxa*, 2344, 17-28.

Theis, K. R., Dheilly, N. M. M., Klassen, J. L., Brucker, R. M., Baines, J. F., Bosch, T. C. G., et al. (2016) Getting the hologenome concept right: An eco-evolutionary framework for hosts and their microbiomes. *bioRxiv*, 38596.

Watanabe, K., Yukuhiro, F., Matsuura, Y., Fukatsu, T., & Noda, H. (2014). Intrasperm vertical symbiont transmission. *Proceedings of the National Academy of Sciences*, 201402476.

Wickham, H. (2016) ggplot2: elegant graphics for data analysis. Springer.

Zilber-Rosenberg, I., and Rosenberg, E. (2008) Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews*, 32(5), 723–735.

FIGURES AND LEGENDS

Figure 1: Developmental consequences of inoculation with species-specific (self) or heterospecific (cross) microbiota in the dung beetle *Onthophagus gazella*. Individuals receiving their own *O. gazella*-specific microbiota are shown in white, while individuals receiving microbiota derived from the congener *O. sagittarus* are shown in grey. **A)** Pupal mass on day 3 during pupation. Cross-inoculated pupae reached a lower mass. **B)** Number of days from egg to pupation. Cross-inoculated *O. gazella* larvae took longer to reach pupation. **C)** Number of days from egg until eclosion of adults. Cross-inoculated larvae took longer to reach adulthood. Numbers below median lines of each box plot represent sample sizes for each group. Numbers above brackets are *P*-values obtained from a Welch's t-test comparing means of the two treatment groups.

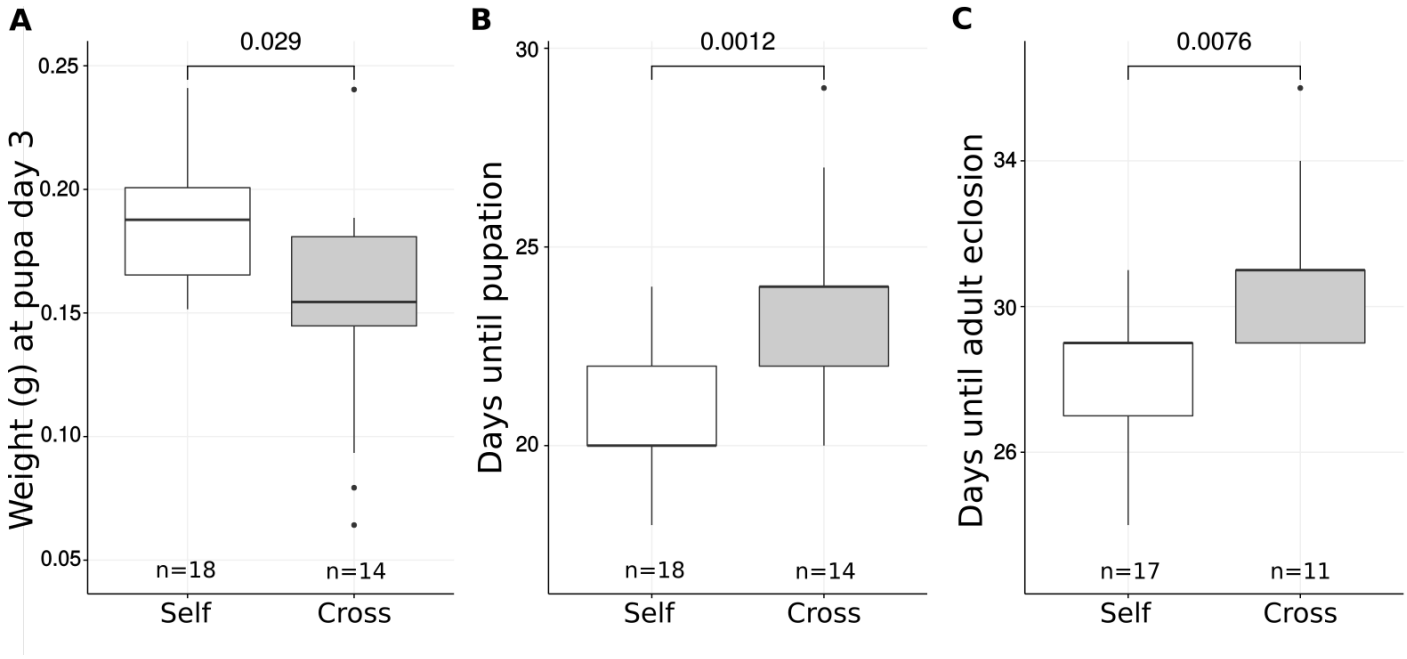


Figure 2: Survival to adulthood of *Onthophagus gazella* and *O. sagittarius* inoculated as larvae with their species-specific microbiota (shown in white) or cross-inoculated with microbiota derived from their respective congener (shown in grey). Cross-inoculated individuals showed lower survival rates in both species, however, this effect was significant only in *O. sagittarius* ($P = 0.0328$) but not *O. gazella* ($P = 0.1904$) where *P*-values represent the results of chi-squared tests. A general relationship between treatment and species was also seen from a chi-square test for independence ($P = 0.00482$).

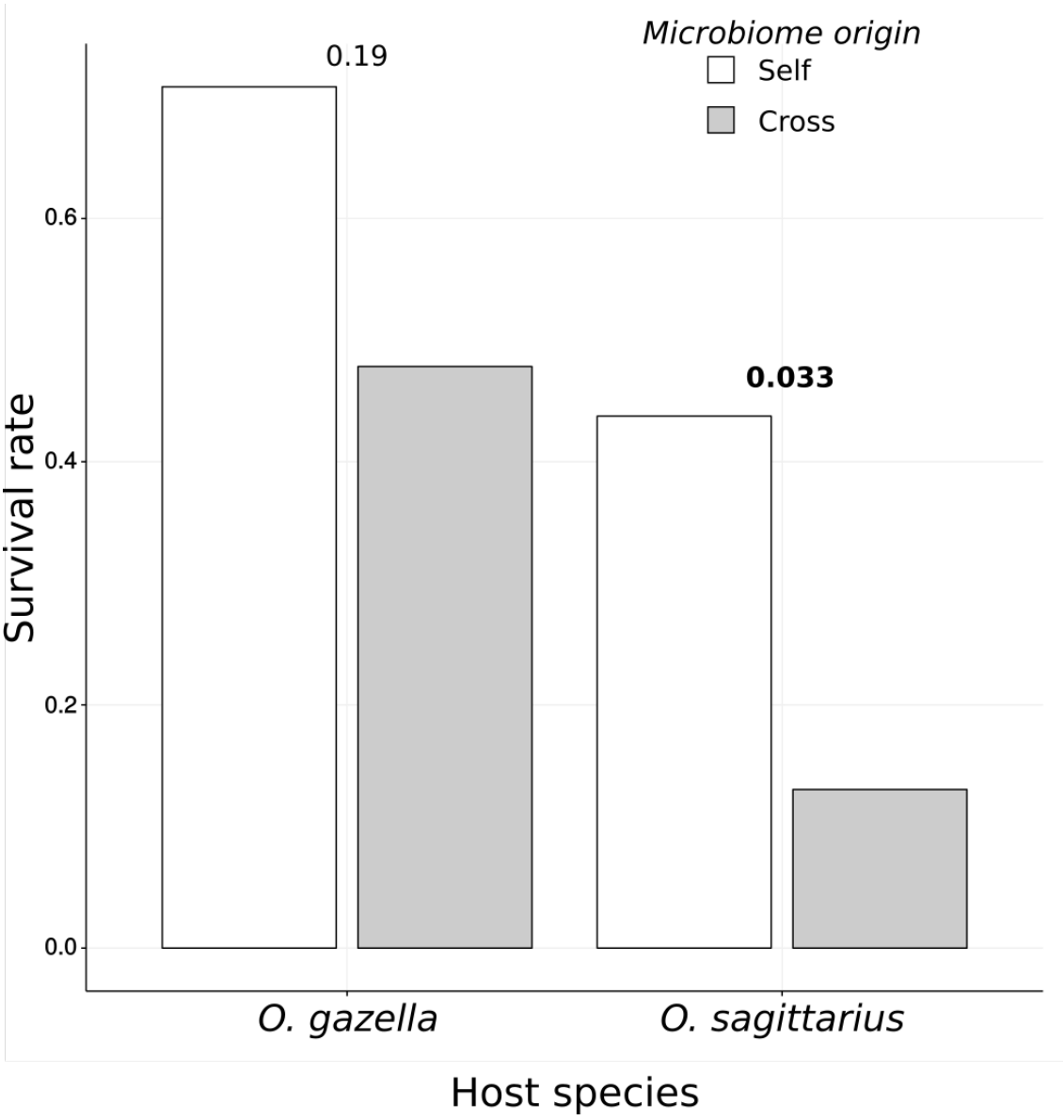
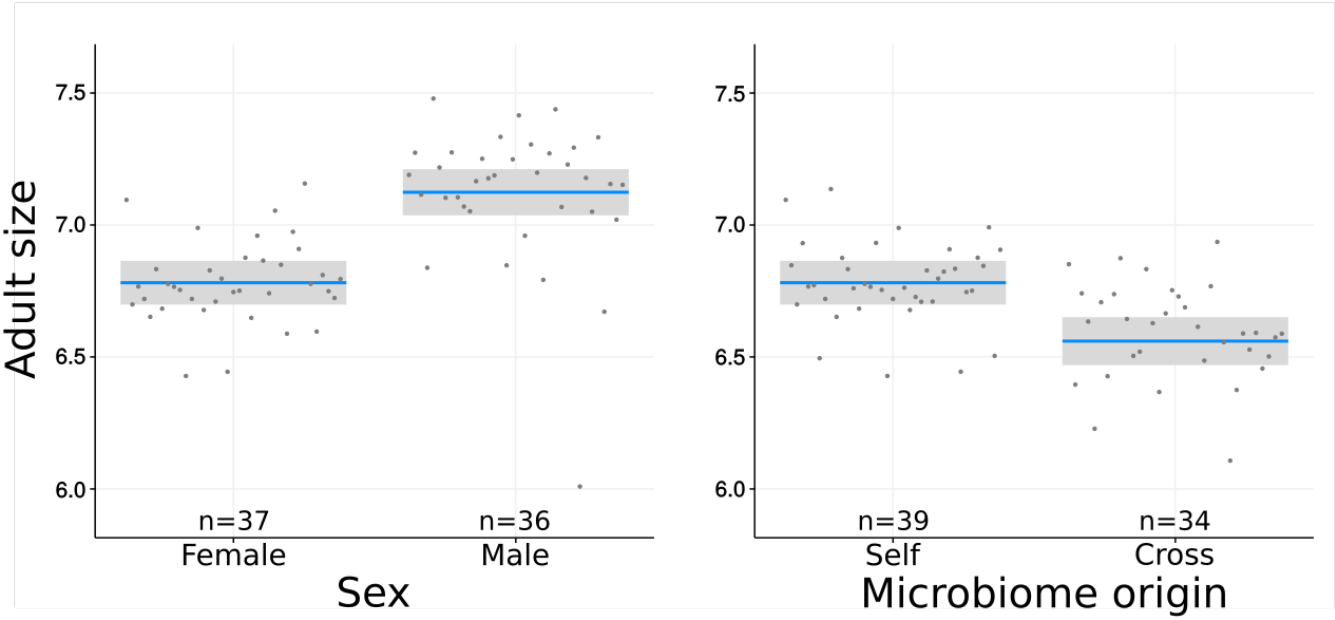


Figure 3: Effect plots showing the predicted values (black lines), and confidence intervals (gray shading) for two explanatory variables when regressed against variation in adult size of F_2 *Onthophagus gazella.* We found that offspring sex, and to a lesser magnitude, microbiota origin significantly explain variation seen in adult size, i.e. after controlling for the effects of all other regressors, male larvae (n = 36) developed to a larger adult size than female larvae (n = 37), and offspring (n = 39) of self-inoculated mothers developed to larger adult sizes than offspring (n = 34) of cross-inoculated mothers. Sex and microbiota origin explained 46.8% of the variation seen in body size when together (model $R^2 = 0.468$), and the relative importance of the two regressors, from the "lmg" decomposition method, was 33.7% and 13.1% respectively (Grömping, 2006).



CHAPTER 3

Reciprocal microbiome transplants differentially rescue fitness in two syntopic dung beetle sister species (Scarabaeidae: Onthophagus)

This chapter has been published as:

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ABSTRACT

1. Microbial symbionts play a crucial role in the development, health, and homeostasis of their hosts. However, the eco-evolutionary conditions shaping these relationships, and the evolutionary scale at which host/microbiome interactions may diverge warrant further investigation, especially in non-model systems. Here we examine the impact of reciprocal gut microbiome transplants between two ecologically very similar, sympatric and syntopic dung beetle sister species.

2. Specifically, we used Onthophagus vacca and O. medius to compare the growth, development, and fitness outcomes of individuals that were either 1) reared in the presence of a microbiome provided by a mother of the same species ("self-inoculated"), or 2) forced to develop with a microbiome derived from a heterospecific mother ("cross-inoculated"), or 3) reared without a maternally-transmitted microbiome.

3. We found that individuals reared in the absence of a maternally-derived gut microbiome incur detrimental changes in survival as well as in several metrics signaling normative

development. Furthermore, such negative effects are only partly rescued through inoculation with a heterologous microbiome.

4. Collectively, our results suggest that inoculation with a species-specific, maternally-transmitted microbiome is critical for normative development, that the significance of maternally-derived microbiota for host survival differs across species, and that the phenotypic outcomes resulting from host-microbiome interactions may diverge even between closely related, ecologically similar host species.

INTRODUCTION

The realization that microbial symbionts are often critical for their host's development, health, and homeostasis has opened diverse novel avenues of investigation into how hosts and their microbiome interact in ways able to shape each other's evolutionary history (Gilbert *et al.*, 2012; McFall-Ngai *et al.*, 2013). In particular, research has demonstrated that exactly what kind of host/microbial associations are able to form, and their respective phenotypic outcomes, may depend greatly on context (e.g., the microbial environment: Vautrin & Vavre, 2009; Schubert *et al.*, 2015; the external, abiotic environment: Corbin *et al.*, 2017; Renoz *et al.*, 2019; Lemoine *et al.*, 2020; and the nutritional environment: Douglas, 2009; Feldhaar, 2011). However, the evolutionary scale at which host/microbiome interactions may diversify remains poorly understood.

Partial progress towards addressing this issue has emerged through the rapidly increasing application of high throughput sequencing, which has facilitated an explosion in large-scale taxonomic comparisons of microbial communities. Such efforts have permitted an assessment of how host relatedness correlates with microbial community similarity (Brooks *et al.*, 2016; Kohl

et al., 2018; Lim & Bordenstein, 2020), or how the microbiome of introduced species may shift to resemble those of native species (Gundale *et al.*, 2016; Parker *et al.*, 2020). In contrast, analyses of the phenotypic outcomes emerging from host-microbiome interactions in the context of host development and health have been limited to a select few systems. Particular foci to date include manipulating the relationships between long-term obligate symbionts and their hosts to assess symbiont function (e.g. aphids and *Buchnera aphidicola*: Moran *et al.*, 1993; Moran & Yun, 2015; Chong & Moran, 2018; leafhoppers: Bennett & Moran, 2013; Koga *et al.*, 2013; Sudakaran *et al.*, 2017), exploring the developmental consequences of microbiome disruption in model systems such as *Drosophila* (Broderick *et al.*, 2014; Bing *et al.*, 2018; Morimoto *et al.*, 2019; Nguyen *et al.*, 2020), and comparing the phenotypic outcomes of host-microbe interactions between distantly related and ecologically divergent taxa (Brooks *et al.*, 2016; Parker *et al.*, 2019). As a consequence, relatively little is known about how early in host diversification, under what types of ecological conditions, and in what systems the phenotypic outcomes emerging from host-microbiome interactions may actually diversify in the wild. Here we investigate the phenotypical significance of host-associated microbiota in two highly ecologically similar, sympatric sister species of dung beetles in the genus *Onthophagus* through a reciprocal transplant experiment.

With over 2,300 extant species found in a variety of habitats and on every continent save Antarctica, *Onthophagus* represents one of the most species-rich and widespread genera in the animal kingdom (Tarasov & Solodovnikov, 2011). Most beetles in this genus utilize dung of mammals for feeding and breeding, excavating tunnels underneath droppings and provisioning dung for offspring in the form of buried ‘brood balls’, each containing a single developing individual (Halffter & Edmonds, 1982). Importantly, *Onthophagus* females vertically transmit

gut microbial communities to their offspring through the *pedestal* – a maternal fecal secretion on which mothers oviposit their eggs within individual brood balls, which is consumed by larvae upon hatching (Estes *et al.*, 2013). Recent work has shown that 1) these pedestal microbiota are crucial for normative development of the host, as depriving juvenile *Onthophagus* beetles of their pedestals leads to marked reductions in adult body size and prolonged development time (Schwab *et al.*, 2016); and that 2) these negative effects are exacerbated under stressful rearing conditions, but can be rescued through re-inoculation with cultured pedestal bacteria (Schwab *et al.*, 2016). In addition to this documented reliance on vertically-transmitted microbiota, the genus *Onthophagus* includes many closely related sister species and species complexes (e.g. Pizzo *et al.*, 2006; Macagno *et al.*, 2011; Breeschoten *et al.*, 2016; Roy *et al.*, 2016; Joaqui *et al.*, 2019), which offer the opportunity to investigate conservation and diversification in host/microbiome interactions over a range of phylogenetic distances, including across recently evolved host species. Here, we explored one such systems - the sister species *Onthophagus medius* and *O. vacca* - to probe the eco-evolutionary contexts that may shape the early stages of diversification in host-microbiome interaction.

O. vacca and *O. medius* are estimated to have last shared a common ancestor in the late Miocene (~8.7 Mya), thereafter undergoing allopatric speciation followed by secondary contact (Roy *et al.*, 2016). To date, both species have broadly overlapping western Palearctic distributions (Roessner *et al.*, 2010) and occupy highly similar ecological niches. While reproductively isolated via postmating/postzygotic barriers, individuals are frequently found in the same locations, feeding in the same dung pads, with no reported local aggregation patterns, and partial phenological overlap (Roy *et al.*, 2016). In this study, we tested whether such closely related and ecologically similar species also share interchangeable microbial symbiont

communities. Using syntopic populations (i.e., populations coexisting in close proximity within the same habitat: Rivas, 1964), we compared growth, development, and fitness outcomes of individuals forced to develop with the pedestal-derived microbiome of the other species ("cross-inoculated") to those reared with their own pedestal ("self-inoculated") and to individuals reared without a pedestal. Based on previous research (Schwab *et al.*, 2016; and see above), we predicted that beetles reared without a pedestal would suffer the greatest reduction in fitness-related growth metrics and survival. Further, we predicted that if divergence in the phenotypic outcomes resulting from host-microbiome interactions already accompanies descent from a common ancestor, cross-inoculation should fail to fully rescue the fitness of developing hosts compared to those receiving their species-specific microbiome.

MATERIALS AND METHODS

Beetle collection and husbandry

Parental *Onthophagus medius* and *O. vacca* were field collected as adults from pastures within the *Pantano della Zittola* peat bog (Isernia province, Italy) in early May 2019, sorted by species, and shipped to Bloomington, IN. All beetles were transferred to species-specific colonies upon arrival in the lab, where they were maintained in a sand/soil mixture at 22ºC and fed antibiotic-free cow dung weekly per Moczek (2006). After a two-week acclimation period in the lab, twenty females per species were provided with *ad libitum* dung and allowed to oviposit for 2-3 weeks until egg depletion, in individual ovipositing containers as detailed below. Brood balls containing developing F1 individuals were harvested and incubated at 22ºC. Once developed to adulthood, individuals of the F1 generation were harvested and housed in monospecific colonies, then subjected to a vernalization protocol similar to that described in Roy *et al.* (2016).

Specifically, temperature in the incubator housing the F1 colonies was lowered weekly by 4°C, from 22°C to 10 °C over the course of three weeks. The colonies were kept at 10°C for one month and then the temperature was again increased to 22°C over a three-week span. Beetles were maintained at 22°C for three additional weeks and subsequently used for experiments as follows.

Experimental design

Seven to ten *O. medius* and *O. vacca* adult females were removed from each colony weekly (total *n O. medius* mothers = 29; *n O. vacca* = 27) and placed individually in plastic ovipositing containers (27 cm X 8 cm X 8 cm) filled with a compacted sand/soil mixture and provided with *ad libitum* dung on top. Brood balls were collected from each ovipositing container after a week and carefully opened with gloved hands. Eggs and pedestals were extracted using sterilized paintbrushes and scalpels, respectively. Eggs were then surface-sterilized with one rinse of 100μL of 1% bleach and 0.1% Triton-X 100 solution, followed by two rinses of 1mL of deionized water. After sterilization, eggs were placed into the center of an artificially constructed brood ball within the well of a twelve-well plate, either on top of an extracted pedestal or on top of the same kind of dung forming the artificial brood ball depending on treatment. Eggs from each species, and mother, were haphazardly assigned to one of three treatment groups: a self-inoculated treatment where each sterile egg was placed back on its own pedestal; a cross-inoculated treatment where eggs were placed on a pedestal from the other species; or an absent treatment where eggs were placed into a well with no pedestal. These six resultant treatment groups were blocked within each 12-well plate so that each plate contained two of each treatment

ID (e.g., two replicates of *O. medius* cross), and their order in each plate was randomized to minimize random within-plate effects.

Plates were then stored at 22ºC for all of development and checked weekly on day 3, day 5 and day 7 following their initial setup to assess animal growth and development stage. After each check, plates were rotated 180º and their placement within the incubator was changed to further minimize any potential microclimatic variation within the incubator. Final sample sizes were for *O. medius*: 82 cross-inoculated, 83 not inoculated, and 77 self-inoculated; and for *O. vacca*: 91 cross-inoculated, 126 not inoculated, and 124 self-inoculated.

Data collection

To assess the impact of our pedestal manipulation protocol on the growth and survival of our experimental animals we collected the following data: 1) mass at the third (and final) larval instar, and the pupal stage, 2) time from hatching of the egg to the onset of the third larval instar, onset of the pupal stage, and adulthood, 3) adult size, and 4) survival to adulthood. Larval mass was measured seven days after an animal was first scored as third instar, as a proxy of each individual's ability to maximize mass gain in the critical rapid growth stage before reaching larval peak (Moczek & Nijhout, 2002; pers. obs.). Pupal mass was measured 48 hours after an individual was first scored as a pupa - this measurement served as an estimate of the final body mass attained by a larva following its gut purge and successful larval to pupal molt. Pupal mass also serves as a close correlate with adult body size in *Onthophagus* (Moczek, 2006). All mass measurements were recorded to the nearest 0.0001g with a Mettler Toledo AL54 (Mettler, Columbus, Ohio, USA) scientific scale. Adult body size was measured as the width of the

pronotum to the nearest 0.01cm using a digital caliper. All individuals were sexed at the pupal stage, when the genital protrusion is clearly visible in males.

Data analysis

All statistical analyses were performed in R v3.5.3 (R Core Team, 2013) and RStudio v1.2.1335 (RStudio Team, 2015) using the packages *car* (Fox *et al.*, 2012), *GGally* (Schloerke *et al.*, 2017), *ggplot2* (Wickham, 2016), *lme4* (Bates *et al.*, 2015), *survival* (Therneau, 2015), *survminer* (Kassambara *et al.*, 2019), and *visreg* (Breheny & Burchett, 2017).

To determine the influence of our pedestal manipulation treatment on the growth, development, and survival of our experimental animals we constructed a series of linear (growth, development), and generalized linear (survival, binomial family) mixed models regressing our measured variables on all possible combinations of the fixed effects of pedestal treatment, species, and sex (included in models considering response variables measured in the pupal and adult stage), as well as their interactions. In each model, plate code was included as a random effect to account for any potential random error introduced by our experimental design. Furthermore, most models also included the unique code of each experimental individual's mother as a random effect, to account for any random variation introduced from maternal line alone. This random effect was not included in our models regressing either size at the third larval instar, or time needed to reach the third larval instar. In these cases, the variance explained by mother's ID was zero, and mother's ID was therefore dropped to avoid overfitting. The regressors in each model were validated using Wald chi-square tests, and non-significant interaction terms were removed. No interactions were ever found to be significant, nor was the

factor sex, so every final model consisted just of the main effects species and pedestal treatment, along with random effects. Standard regression diagnostics were performed on each final model.

Finally, survival curves for each of our six treatment groups were calculated using the Kaplan-Meier estimator (Kaplan & Meier, 1958), and the resultant curves were compared using the nonparametric log-rank test.

RESULTS

Using a reciprocal transplant experiment, we sought to assess whether two dung beetle sister species with a long history of sympatry, syntopy, and broadly overlapping ecological niches utilize interchangeable gut microbiomes, or alternatively may have diverged in host/microbiome interactions. Our results support the latter hypothesis, as detailed below.

Cross-inoculation differentially rescues survival in Onthophagus vacca *and* O. medius

Our three pedestal treatments had marked effects on survival in both focal species. Specifically, when reared without a pedestal, individuals survived at the lowest rate, and showed the most precipitous early decline in survival compared to self-inoculated individuals, which survived at a significantly higher rate and did not experience a comparable drop in survival early on (Table 1; Fig. 1). However, both *O. vacca* and *O. medius* were differentially affected by cross-inoculation: when cross-inoculated with *O. vacca* pedestals, *O. medius* survived at intermediate rates, significantly different from both self-inoculation (log-rank test: $p = 0.028$) and absence of a pedestal (log-rank test: $p = 0.027$) (Fig. 1B). In contrast, in *O. vacca* cross-inoculation with *O. medius* pedestals restored survival sufficiently that it became significantly different only from that of pedestal-free individuals, but statistically indistinguishable from *O. vacca* inoculated with

O. vacca pedestals (log-rank test: $p = 0.5$) (Fig. 1A). Combined, these results suggest that cross-inoculation with a heterologous pedestal is sufficient to largely restore survival in *O. vacca*, but not in *O. medius*.

Pedestal-free rearing reduces growth, delays development, and is only partly reversed through inoculation with a heterologous microbiome

In both *O. vacca* and *O. medius*, our pedestal manipulations negatively impacted a range of growth and developmental metrics tied to fitness in insects (Moczek, 1998; Kingsolver & Huey, 2008). Specifically, while *O. vacca* generally developed faster and achieved higher larval mass, in both species the absence of a maternally derived pedestal significantly prolonged development and lowered mass as measured at day 7 of the third (= last) larval instar (Table 1; Fig. 2A&B). Cross-inoculation with a heterologous pedestal partly reversed a subset of these effects to roughly comparable degrees in both species. That is, we found that cross-inoculated animals reached the third larval instar faster than pedestal-free animals, and at a rate indistinguishable from self-inoculated animals in both *O. vacca* (Wald chi-square: $X^2 = 1.16$, $p = 0.28$) and *O. medius* (Wald chi-square: $X^2 = 0.014$, $p = 0.91$) (Table 1; Fig. 2A). However, this acceleration of development caused by cross-inoculation disappeared during later timepoints. Cross-inoculated *O. vacca* (Wald chi-square: $X^2 = 0.027$, $p = 0.87$) and *O. medius* (Wald chi-square: $X^2 = 2.64$, $p = 0.10$) both reached the pupal stage at the same rate as pedestal-free animals, and slower than self-inoculated individuals (Table 1; Fig. 2C). Similarly, cross-inoculated *O. vacca* (Wald chi-square: $X^2 = 0.601$, $p = 0.44$) and *O. medius* (Wald chi-square: $X^2 = 0.927$, $p = 0.34$) completed development at the same reduced rate as pedestal-free animals (Table 1; Fig. 2D).

Furthermore, we found an overall significant effect of our pedestal manipulation on mass as measured at day 7 of the third larval instar (Wald chi-square: $X^2 = 7.034$, $p = 0.03$) (Table 1). Specifically, in both species cross-inoculation reduced L3 mass by an extent similar to pedestal deprivation ($\beta = -0.0029 \pm 0.0051$), while self-inoculation resulted in individuals reaching comparatively greater L3 mass ($\beta = 0.0087 \pm 0.0046$) (Table 1, Fig. 2B). Together, these results suggest that inoculation with a heterologous pedestal and corresponding microbiota is insufficient to fully restore growth and development time during larval ontogeny of either species. Finally, despite these differences found in developmental rate and mass during early development, we failed to find a significant effect of either species or pedestal treatment on both pupal mass and adult body size (as shown by non-significant results for these two factors in Table 1).

DISCUSSION

We investigated whether two ecologically overlapping and geographically co-occurring dung beetle sister species utilize interchangeable gut microbiomes. Using a reciprocal transplant experiment, we found that individuals reared in the absence of a maternally-derived gut microbiome suffer reduced survival as well as detrimental changes in several fitness-relevant developmental metrics. Furthermore, we found that such negative effects are only partly rescued through inoculation with a heterologous microbiome (i.e., a pedestal derived from a heterospecific mother), suggesting that developmentally-significant divergences in the phenotypic outcomes resulting from host-microbiome interactions may already manifest during sister species formation and in spite of highly similar ecological conditions. Below we discuss the most significant implications of our results.

Inoculation with a species-specific, maternally-transmitted microbiome is critical for normative development

In line with previous research (Schwab *et al.*, 2016), we found that animals reared without access to a pedestal performed worse than animals provided their own, species-specific pedestal in a host of fitness-relevant developmental metrics (Fig. 2; Table 1). Additionally, while overall *O. medius* developed slower than *O. vacca*, both pedestal-free and cross-inoculated animals took longer to reach the pupal and adult stages than self-inoculated individuals, revealing that inoculation with a heterologous pedestal slows development in both species. Interestingly, only pedestal-free, but not cross-inoculated animals showed a significant increase in the time needed to reach the final (= third) larval instar when compared to self-inoculated animals (Fig. 2A; Table 1). That is, cross-inoculated individuals developed at the same pace as self-inoculated individuals up until the third larval instar, but slowed down significantly thereafter, ultimately reaching the pupal and adult stages at the same rate as pedestal-free animals. Additionally, cross-inoculated animals had significantly lower mass seven days into the final larval instar than self-inoculated larvae (Fig. 2B; Table 1). Our data therefore demonstrate that cross-inoculation has little impact on developmental rate during the early larval stages, but does significantly slow growth in the third larval instar - a period critical for rapid mass gain in *Onthophagus* (Moczek & Nijhout, 2002) - possibly leading to subsequent developmental delays as cross-inoculated animals must spend more time feeding as larvae in order to gain sufficient mass for the onset of pupation to occur (Shafiei *et al.*, 2001). These results thus lend further support to the idea of dung beetle microbiota as a host species-specific nutritional symbiont (Estes *et al.*, 2013; Schwab *et al.*, 2016; Shukla *et al.*, 2016; Parker *et al.*, 2019).

Host species differ in their reliance on maternally-transmitted microbiome for survival

Pedestal-free rearing not only reduced growth and delayed development, but also substantially affected survival rates. That is, while overall *O. vacca* survived at higher rates than *O. medius*, pedestal-free rearing severely reduced survival during development in both species (Fig. 1; Table 1). Previous research showed a similar reduction in survival in pedestal-free individuals of a different *Onthophagus* species, but only when reared under stressful environmental conditions (high desiccation stress and temperature fluctuations: Schwab *et al.*, 2016). By comparison, in *O. vacca* and *O. medius* the negative effects of pedestal removal were obvious even under the relatively benign rearing conditions used in this study. At the same time, in both *O. vacca* and *O. medius* cross-inoculation improved survival as compared to pedestal-free rearing. However, while *O. vacca* reared with heterologous pedestals showed survival rates statistically indistinguishable from those reared with their own pedestal, *O. medius* receiving a heterologous pedestal survived at a rate higher than pedestal-free, but still lower than self-inoculated animals (Fig. 1B). Our pedestal exchange experiment therefore provides further support for differential, host species-specific reliance on pedestal microbiota (also see Parker *et al.*, 2019). In particular, our results suggest that even though reliance on maternally-transmitted microbiota for normative host development may be a general feature in *Onthophagus*, different host species within this genus may nevertheless diverge in the extent of this reliance.

Divergence in the phenotypic outcomes resulting from host-microbiome interactions is detectable even in closely related, ecologically similar species

To date few studies have investigated the potential for divergence in the phenotypi consequences of host-microbiome interactions across host species (Brooks *et al.*, 2016; Sudakaran *et al.*, 2017). Among dung beetles, such putative interspecific differentiation was detected by swapping pedestals between *Onthophagus sagittarius* and *Digitonthophagus gazella* (Parker *et al.*, 2019). While both are tunneling dung beetle species belonging to the tribe Onthophagini, they are phylogenetically much more distant than the sister species used in the present study (37 MYA: Breeschoten *et al.*, 2016). Further, *O. sagittarius* and *D. gazella* derive from different continents and have only had a very recent history of sympatry following artificial introductions into Australia in the 1970s as part of a biocontrol program (Edwards, 2007). Reciprocal microbiome transplants across these focal species similarly affected developmental metrics and survival in a host-specific manner. Yet, diversification of distantly-related hosts in their reliance onto non-interchangeable microbial communities could simply be a product of their great phylogenetic distance and biogeographic separation. By contrast, our finding that pedestal cross-inoculation between *Onthophagus vacca* and *O. medius* fails to fully rescue the fitness of developing individuals suggests that divergence in the phenotypic outcomes of host/microbiome interactions may indeed already accompany descent from a common ancestor, and manifest over much shorter evolutionary time periods. Moreover, these sister species appear to rely on non-interchangeable microbiomes *despite* their long history of sympatry/syntopy and broadly overlapping autecologies (Roy *et al.*, 2016), raising questions as to exactly what evolutionary and ecological dynamics may have driven, and are now maintaining, host-specific microbiome divergences.

It is currently hypothesized that *O. vacca* and *O. medius* speciated in allopatry, and only subsequently established their present-day sympatric ranges as a result of secondary contact

(Rossner *et al.*, 2010; Roy *et al.*, 2016). Stochastic (e.g., priority and founder effects) or deterministic forces (e.g., host selection and environmental pressure), both of which have the potential to significantly impact microbiome assemblies (Maignien *et al.*, 2014; Schmidt *et al.*, 2015; Vecchi *et al.*, 2018; Parker *et al.*, 2020), may therefore have shaped distinct host/microbiome interactions already during the allopatric stage of species formation. If true, the results of our study reflect a relatively deep divergence, established during speciation and then maintained throughout secondary contact. In addition, divergence in the phenotypic outcomes of host-microbiome interactions might also have arisen, or been emphasized, once the two species re-established contact. In this scenario, the establishment of diverging host-symbiont relationships - possibly combined with differential microhabitat specializations - may have facilitated the maintenance of the two sister species in syntopy, avoiding competitive exclusion (Levin, 1970; Schoener, 1974; Scriven *et al.*, 2016). Finally, given that 1) *O. vacca* and *O. medius* can interbreed in captivity, but form low fitness hybrids (Roy *et al.*, 2016), and that 2) research in other insects has established that such post-mating hybrid lethality can be attributed directly to the maternally-transmitted microbiome (Brucker & Bordenstein, 2013), it is also possible that reliance on a non-interchangeable microbiome may contribute to sympatric speciation via reinforcement (i.e., selection against hybridization) in these sister species. If true, *Onthophagus vacca* and *O. medius* would join a growing list of examples illustrating the potential of microbial symbionts to contribute to speciation of their hosts (Sharon *et al.*, 2010; Lizé *et al.*, 2013; Morimoto *et al.*, 2017; Leftwitch *et al.*, 2018). Further studies are needed to confirm or discard this possibility.

CONCLUSIONS

Progressing beyond taxonomic descriptions of the microbiome towards a more comprehensive understanding of the emergent properties of the complex interactions between microbial symbionts and their hosts, and of the ecological and evolutionary conditions shaping these relationships, remains a crucial goal, especially in non-model systems. Previous work documented that dung beetle species may associate with non-interchangeable microbiota (Parker *et al.*, 2019), yet the phylogenetic scope and ecological conditions that facilitate such divergences remained to be characterized. Here we have shown that sister species may rely on non-interchangeable microbiomes to support their development and enhance their survival. Importantly, our observations suggest that such disparate, non-equivalent host-microbiota associations may be maintained despite a long history of coexistence in the same geographical areas and overlapping host autecologies. Vertical transmission appears as perhaps the most plausible strategy to maintain such associations, though host-specific differential horizontal acquisition of selected strains from the environment can at present not be excluded as an alternate, or additional mechanism (Moran & Sloan, 2015; Shapira, 2016). Further investigations into the phenotypic significance of maternally-transmitted microbial symbionts of closely related host species in both sympatry and allopatry, coupled with an analysis of their potential for the maintenance of the hosts' reproductive barriers, may shed more light onto how hosts and their microbiome interact in ways able to shape each other's evolutionary history. Additionally, molecular-based insights into the composition and vertical transmission of pedestal-inoculated microbiota would greatly help elucidate the functions provided by microbial symbionts, and whether microbiome divergences may precede, parallel, or follow speciation events of *Onthophagus* hosts.

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REFERENCES

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software,* **67**, 1–48.

Bennett, G.M. & Moran, N.A. (2013) Small, smaller, smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biology and Evolution*, **5**, 1675–1688.

Bing, X., Gerlach, J., Loeb, G. & Buchon, N. (2018). Nutrient-dependent impact of microbes on *Drosophila suzukii* development. *MBio*, **9**, e02199-17.

Breeschoten, T., Doorenweerd, C., Tarasov, S. & Vogler, A.P. (2016) Phylogenetics and biogeography of the dung beetle genus *Onthophagus* inferred from mitochondrial genomes. *Molecular Phylogenetics and Evolution*, **105**, 86–95.

Breheny, P. & Burchett, W. (2017) Visualization of regression models using visreg. *The R Journal*, **9**, 56–71.

Brooks, A.W., Kohl, K.D., Brucker, R.M., van Opstal, E.J. & Bordenstein, S.R. (2016) Phylosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. *PLOS Biology*, **14**, e2000225.

Broderick, N. A., Buchon, N. & Lemaitre, B. (2014). Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. *MBio*, **5**, e01117-14.

Brucker, R.M. & Bordenstein, S.R. (2013) The hologenomic basis of speciation. *Science*, **466**, 667–669.

Chong, R.A. & Moran, N.A. (2018) Evolutionary loss and replacement of *Buchnera*, the obligate endosymbiont of aphids. *ISME Journal*, **12**, 898–908.

Corbin, C., Heyworth, E.R., Ferrari, J. & Hurst, G.D.D. (2017) Heritable symbionts in a world of varying temperature. *Heredity*, **118**, 10–20.

Douglas, A.E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, **23**, 38–47.

Edwards, P. (2007) *Introduced dung beetles in Australia 1967-2007: current status and future directions*. Dung Beetles for Landcare Farming Committee, Sinnamon Park, Qld, Australia.

Estes, A.M., Hearn, D.J., Snell-Rood, E.C., Feindler, M., Feeser, K., Abebe, T., Dunning Hotopp J.C., Moczek, A.P. (2013) Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS ONE*, **8**, 1–15.

Feldhaar, H. (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology*, **36**, 533–543.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., Firth, D., Friendly, M., Gorjanc, G., Graves, S., Heiberger, R., Laboissiere, R., Monette, G. & Murdoch, D. (2012) *Package 'car'*. R Foundation for Statistical Computing, Vienna, Austria.

Gilbert, S.F., Sapp, J. & Tauber, A.I. (2012) A symbiotic view of life: we have never been individuals. *The Quarterly Review of Biology*, **87**, 325–341.

Gundale, M.J., Almeida, J.P., Wallander, H., Wardle, D.A., Kardol, P., Nilsson, M.C., Fajardo, A., Pauchard, A., Peltzer, D.A., Ruotsalainen, S., Mason, B. & Rosenstock, N. (2016) Differences in endophyte communities of introduced trees depend on the phylogenetic relatedness of the receiving forest. *Journal of Ecology*, **104**, 1219–1232.

Halffter, G. & Edmonds, W.D. (1982) *The nesting behavior of dung beetles (Scarabaeinae). An ecological and evolutive approach.* Publication 10, Instituto de Ecologia, Mexico, D.F.

Joaqui T., Moctezuma V., Sánchez-Huerta J.L. & Escobar F. (2019) The *Onthophagus fuscus* (Coleoptera: Scarabaeidae) species complex: an update and the description of a new species. Zootaxa, **4555**, 151– 186.

Kaplan, E.L. & Meier, P. (1958) Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, **53**, 457–481.

Kassambara, A., Kosinski, M., Biecek, P. & Fabian, S. (2019) *survminer: drawing survival curves using "ggplot2".* R package version 0.4.4.

Kingsolver, J.G. & Huey, R.B. (2008) Size, temperature, and fitness: three rules. *Evolutionary Ecology Research*, **10**, 251–268.

Koga, R., Bennett, G.M., Cryan, J.R. & Moran, N.A. (2013) Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. *Environmental Microbiology*, **15**, 2073–2081.

Kohl, K.D., Dearing, M.D. & Bordenstein, S.R. (2018) Microbial communities exhibit host species distinguishability and phylosymbiosis along the length of the gastrointestinal tract. *Molecular Ecology*, **27**, 1874–1883.

Leftwich, P. T., Hutchings, M. I. & Chapman, T. (2018). Diet, Gut Microbes and Host Mate Choice: Understanding the significance of microbiome effects on host mate choice requires a case by case evaluation. *BioEssays*, **40**, 1800053.

Levin, S.A. (1970) Community equilibria and stability, and an extension of the competitive exclusion principle. *The American Naturalist*, **104**, 413–423.

Lemoine, M.M., Engl, T. & Kaltenpoth, M. (2020) Microbial symbionts expanding or constraining abiotic niche space in insects. *Current Opinion in Insect Science*, **39**, 14–20.

Lim, S.J. & Bordenstein, S.R. (2020) An introduction to phylosymbiosis. *Proceedings of the Royal Society B: Biological Sciences*, **287**, 20192900.

Lizé, A., McKay, R. & Lewis, Z. (2013) Gut microbiota and kin recognition. *Trends in Ecology & Evolution*, **28**, 325–326.

Macagno, A.L.M., Pizzo, A., Rolando, A. & Palestrini, C. (2011) Size and shape interspecific divergence patterns partly reflect phylogeny in an *Onthophagus* species-complex (Coleoptera: Scarabaeidae). *Zoological Journal of the Linnean Society*, **162**, 482–498.

Maignien, L., DeForce, E.A., Chafee, M.E., Eren, A.M. & Simmons, S.L. (2014) Ecological succession and stochastic variation in the assembly of *Arabidopsis thaliana* phyllosphere communities. *MBio*, **5**, e00682–13.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders J.G., Tautz, D., & Wernegreen, J.J. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 3229–3236.

Moczek, A.P (1998) Horn polyphenism in the beetle *Onthophagus taurus*: larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. *Behavioral Ecology*, ***9***, 636–641.

Moczek, A.P. (2006) Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *The American Naturalist*, **168**, 711–729.

Moczek, A.P. & Nijhout, H.F. (2002) Developmental mechanisms of threshold evolution in a polyphenic beetle. *Evolution & Development*, **4**, 252–264.

Moran, N.A., Munson, M.A., Baumann, P. & Ishikawa, H. (1993) A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **253**, 167–171.

Moran, N.A. & Sloan, D.B. (2015) The hologenome concept: helpful or hollow? *PLoS Biology*, **13**, 1–10.

Moran N.A. & Yun, Y. (2015) Experimental replacement of an obligate insect symbiont. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 2093–2096.

Morimoto, J., Simpson, S.J. & Ponton, F. (2017) Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, **13**, 20160966.

Morimoto, J., Nguyen, B., Tabrizi, S.T., Lundbäck, I., Taylor, P.W., Ponton, F. & Chapman, T.A. (2019) Commensal microbiota modulates larval foraging behaviour, development rate and pupal production in *Bactrocera tryoni*. *BMC Microbiology*, **19**, 1–8.

Nguyen, B., Than, A., Dinh, H., Morimoto, J. & Ponton, F. (2020) Parental microbiota modulates offspring development, body mass and fecundity in a polyphagous fruit fly. *Microorganisms*, **8**, 1289.

Parker, E.S., Dury, G.J. & Moczek, A.P. (2019) Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles. *Ecological Entomology*, **44**, 274–282.

Parker, E.S., Newton, I.L.G. & Moczek, A.P. (2020) (My microbiome) would walk 10,000 miles: maintenance and turnover of microbial communities in introduced dung beetles. *Microbial Ecology*, **80**, 435–446.

Pizzo, A., Roggero, A., Palestrini, C., Cervella, P., Del Pero, M. & Rolando, A. (2006) Genetic and morphological differentiation patterns between sister species: the case of *Onthophagus taurus* and *Onthophagus illyricus* (Coleoptera, Scarabaeidae). *Biological Journal of the Linnean Society*, **89**, 197–211.

R Core Team (2013) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Renoz, F., Pons, I. & Hance, T. (2019) Evolutionary responses of mutualistic insect–bacterial symbioses in a world of fluctuating temperatures. *Current Opinion in Insect Science*, **35**, 20–26.

Rivas, L.R. (1964) A reinterpretation of the concepts "Sympatric" and "Allopatric" with proposal of the additional terms "Syntopic" and "Allotopic". *Systematic Zoology*, **13**, 42–43.

RStudio Team (2015) *RStudio: integrated development for R*. RStudio, Inc., Boston, MA.

Roessner, E., Schoenfeld, J. & Ahrens, D. (2010) *Onthophagus* (*Palaeonthophagu*s) *medius* (Kugelann, 1792) – a good western palaearctic species in the *Onthophagus vacca* complex (Coleoptera: Scarabaeidae: Scarabaeinae: Onthophagini). *Zootaxa*, **2629**, 1–28.

Roy, L., Bon, M.C., Cesarini, C., Serin, J. & Bonato, O. (2016) Pinpointing the level of isolation between two cryptic species sharing the same microhabitat: a case study with a scarabaeid species complex. *Zoologica Scripta*, **45**, 407–420.

Schloerke, B., Cook, D., Larmarange, J. Briatte, F., Marbach, M., Thoen, E., Elberg, A., Toomet, O., Crowley, J., Hofmann, H. & Wickham, H. (2017) *GGally: Extension to 'ggplot2'*. R Package Version 1.3.1.

Schmidt, V.T., Smith, K.F., Melvin, D.W. & Amaral-Zettler, L.A. (2015) Community assembly of a euryhaline fish microbiome during salinity acclimation. *Molecular Ecology*, *24*, 2537–2550.

Schoener, T.W. (1974) Resource partitioning in ecological communities. *Science*, **185**, 27–39.

Schubert, A.M., Sinani, H. & Schloss, P.D. (2015) Antibiotic-induced alterations of the murine gut microbiota and subsequent effects on colonization resistance against *Clostridium difficile*. *MBio*, **6**, e00974-15

Schwab, D.B., Riggs, H.E., Newton, I.L.G. & Moczek, A.P. (2016) Developmental and ecological benefits of the maternally transmitted microbiota in a dung beetle. *The American Naturalist*, **188**, 679–692.

Scriven, J.J., Whitehorn, P.R., Goulson, D. & Tinsley, M.C. (2016) Niche partitioning in a sympatric cryptic species complex. *Ecology and Evolution*, **6**, 1328–1339.

Shafiei, M., Moczek, A.P. & Nijhout, H.F. (2001) Food availability controls onset of metamorphosis in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Physiological Entomology*, **26**, 173–180.

Shapira, M. (2016) Gut microbiotas and host evolution: scaling up symbiosis. *Trends in Ecology and Evolution*, **31**, 539–549.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I. & Rosenberg, E. (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*.

Proceedings of the National Academy of Sciences of the United States of America, **107**, 20051–20056.

Shukla, S.P., Sanders, J.G., Byrne, M.J. & Pierce N.E. (2016) Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular Ecology*, **25**, 6092–6106.

Sudakaran, S., Kost, C. & Kaltenpoth, M. (2017) Symbiont acquisition and replacement as a source of ecological innovation. *Trends in Microbiology*, **25**, 375–390.

Tarasov, S.I. & Solodovnikov, A.Y. (2011) Phylogenetic analyses reveal reliable morphological markers to classify mega-diversity in Onthophagini dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae). *Cladistics*, **27**, 490–528.

Therneau, T. (2015) *A Package for Survival Analysis in R*. R package version 2.38.

Vautrin, E. & Vavre, F. (2009) Interactions between vertically transmitted symbionts: cooperation or conflict? *Trends in Microbiology*, **17**, 95–99.

Vecchi, M., Newton, I.L.G., Cesari, M., Rebecchi, L. & Guidetti, R. (2018) The microbial community of tardigrades: environmental influence and species specificity of microbiome structure and composition. *Microbial Ecology*, **76**, 467–481.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*. Springer-Verlag, New York.

FIGURES AND LEGENDS

Fig. 1: Effect of pedestal manipulation on survival of *Onthophagus vacca* and *O. medius*. Survival curves of *O. vacca* (left) and *O. medius* (right) larvae who received their own pedestal (self-inoculated), the other species' pedestal (cross-inoculated), or no pedestal (none). Curves were calculated using the Kaplan-Meier estimator, and the distributions of the curves were compared using non-parametric log-rank test. In both species, self-inoculated animals showed the greatest survival rate throughout the course of the experiment, while animals receiving no pedestal showed the lowest. In *O. vacca*, cross-inoculation rescued fitness as compared to the no pedestal treatment to the extent that final survival rate was indistinguishable from self-inoculated animals. In *O. medius* cross-inoculation also improved survival as compared to no pedestal, but not to the extent seen in the self-inoculated treatment.

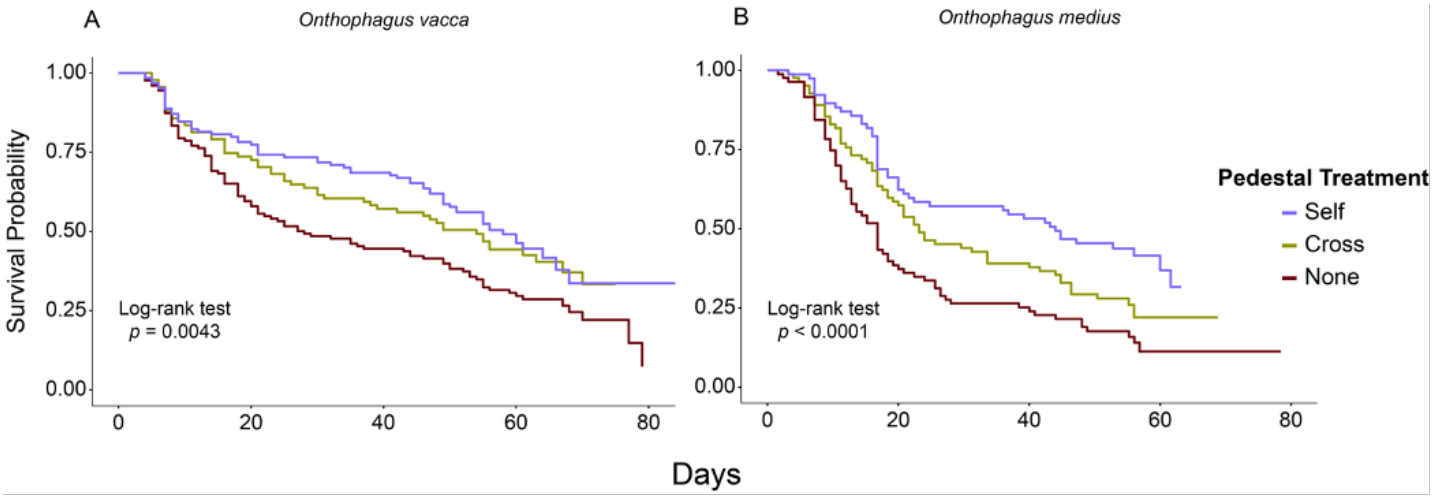

Fig. 2: Effect of pedestal manipulation on developmental metrics. Effects plots showing the estimated influence of pedestal treatment and species on A) days until the third (and final) larval instar (n = 333), B) Mass in grams at day 7 of the third larval instar (n = 304), C) days until the pupal stage (n = 228), and D) total developmental time as days until adult eclosion (n = 195). All plots were derived from a linear mixed model containing the factors pedestal treatment and species, as well as the random factors of plate code and identity of mother (omitted from A, B because the variance of this effect was zero). In general, *Onthophagus vacca* develop faster, and are smaller during the larval stage than *O. medius*. Furthermore, self-inoculated animals have the fastest development and are the heaviest as larvae, while animals receiving no pedestal develop the slowest and are the lightest. Cross-inoculated animals are generally intermediate between these groups. Points indicate partial residuals, and horizontal colored lines indicate predicted values in each plot.

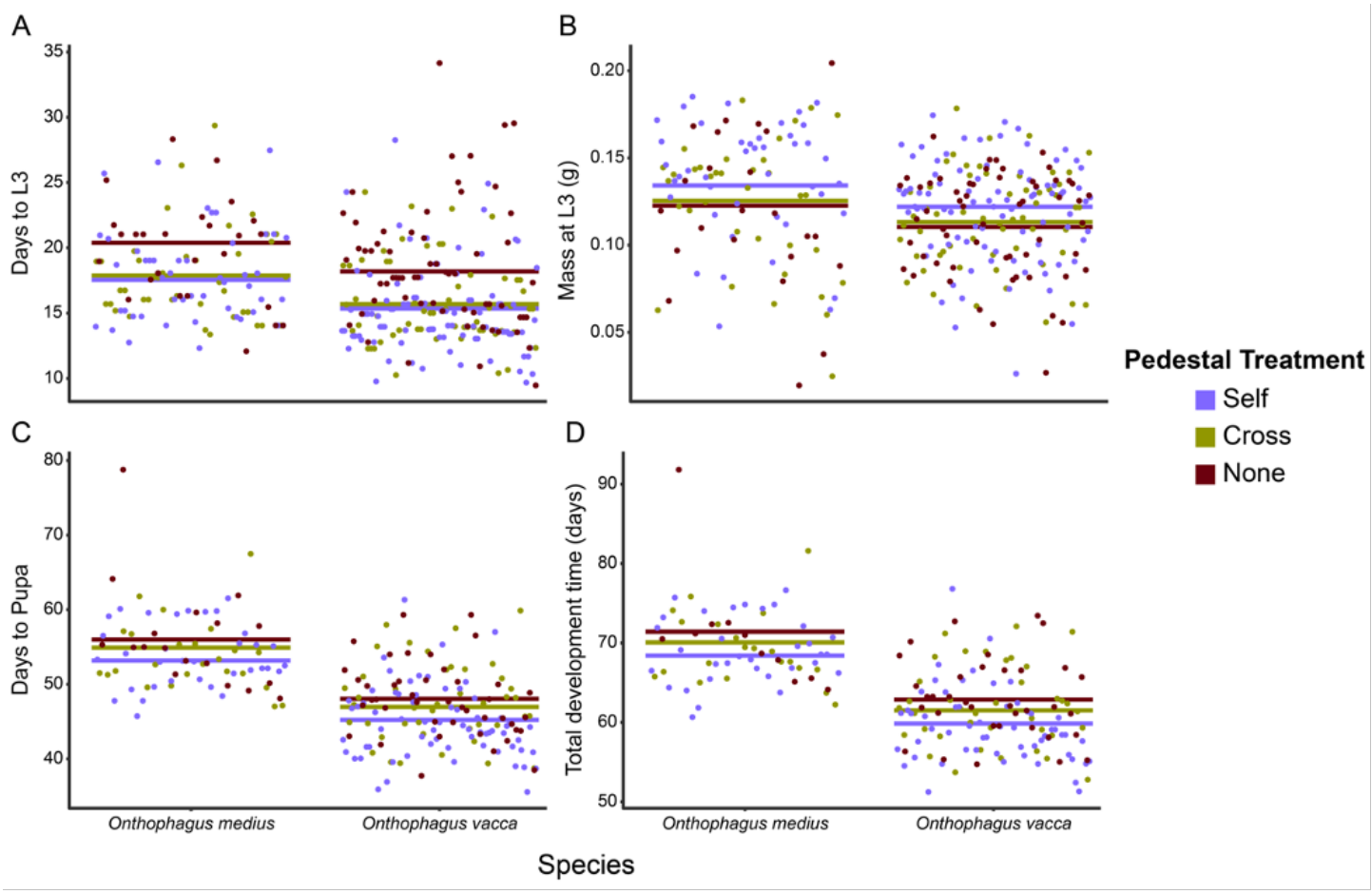
A
B
C
D
Days to L3
Mass at L3 (g)
Days to Pupa
Total development time (days)
Onthophagus medius
Onthophagus vacca
Species
Pedestal Treatment
Self
Cross
None

Table 1: Coefficients of mixed models testing for the significance of pedestal treatment, and species on fitness-related developmental metrics. 12-well plate code, and maternal ID was used in a random effect in each model (except for days to L3 and larval weight, where maternal ID was dropped - see Methods for details). All non-significant interactions were removed. Rows show Chi-square (X^2) test statistics values, the resulting test probabilities, and estimated effect sizes plus or minus standard error for each response variable. Notations in parentheses following β and standard error (SE) estimates reflect the change in value from one category to another (e.g. -2.19±0.51 (*vacca*) means that "days to L3" decrease by 2.19±0.51 for *O. vacca* compared to *O. medius*).

		species	**pedestal**
Days to L3	X^2	17.22	32.55
	p	**<.001**	**<.001**
	$\beta \pm SE$	-2.19±0.51 (*vacca*)	2.51±0.56 (None), -0.33±0.5 (Self)
Mass at L3	X^2	5.64	7.03
	p	**0.018**	**0.03**
	$\beta \pm SE$ *(g)*	-0.012±0.0051 (*vacca*)	-0.0029±0.0051 (None), 0.0087±0.0046 (Self)
Days to pupa	X^2	48.44	9.94
	p	**<.001**	**0.007**
	$\beta \pm SE$	-7.96±1.14 (*vacca*)	1.086±1.023 (None), -1.75±0.91 (Self)
Pupal mass	X^2	0.053	4.48
	p	0.82	0.11
	$\beta \pm SE$	0.0008 ± 0.0033 (*vacca*)	0.0003 ± 0.0038 (None),

			0.0061±0.0033 (Self)
Total development time	X^2	31.36	8.19
	p	**<.001**	**0.02**
	$\beta \pm SE$ *(days)*	-8.55±1.53 (*vacca*)	1.35±1.18 (None), -1.67±1.01 (Self)
Adult size	X^2	0.983	4.34
	p	0.32	0.11
	$\beta \pm SE$	-0.082±0.083 (*vacca*)	0.029 ±0.086 (None), 0.14±0.074 (Self)
Survival	X^2	5.54	22.59
	p	**0.019**	**<.001**
	$\beta \pm SE$ *(prob.)*	0.33±0.31 (*vacca*)	0.68±0.26 (None), 0.41±0.24 (Self)

CHAPTER 4

Don't stand so close to me: microbiota-facilitated enemy release dynamics in introduced *Onthophagus taurus* dung beetles.

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ABSTRACT

Microbial symbionts can influence their hosts in stunningly diverse ways. Emerging research suggests that an underappreciated facet of these relationships is the influence microbes can have on their host's responses to novel, or stressful, environmental conditions. We sought to address these and related questions in populations resulting from the recent introduction and subsequent rapid range expansion of *Onthophagus taurus* dung beetles. Specifically, we manipulated both microbial communities and rearing temperature to detect signatures of developmental and life history differentiation in response to the local thermal conditions in two populations derived from the southern-most (Florida) and northern-most (Michigan) extremes of the exotic Eastern U.S. range of *O. taurus*. We then sought to determine the contributions, if any, of host-associated microbiota to this differentiation. We found that when reared under common garden conditions individuals from Florida and Michigan populations differed significantly in developmental performance measures and life history traits, consistent with population divergence. At the same time, and contrary to our predictions, we failed to find support for the hypothesis that animals perform better if reared at temperatures that match their location of origin, and that performance

differences may be mediated by host-associated microbiota. Instead, we found that microbiome swapping across host populations improved developmental performance in both populations, consistent with enemy release dynamics. We discuss the implications of our results for our understanding of the rapid spread of exotic *O. taurus* through the Eastern US, and the significance of symbiosis in host responses to novel environmental conditions more broadly.

INTRODUCTION

Responding to changing environmental conditions requires organisms to either plastically shift patterns of phenotypic expression within a lifetime or undergo adaptive evolution across multiple generations (West-Eberhard, 2003; Barrett and Schluter, 2008). The individual contributions of these two mechanisms as well as their potential synergistic effects are of particular interest as we consider the impacts of anthropogenic climate change, especially as they relate to crucial ecosystem service providers (Mooney et al., 2009; Merilä and Hendry, 2014; Kingsolver and Buckley, 2017). However, what is less well understood is how microbial symbionts might ameliorate both plastic and adaptive responses of their hosts when confronted with novel, or stressful, environmental conditions. Such microbiome-mediated ecological adaptation has recently been hypothesized to be a relatively common occurrence (Sudakaran et al., 2017), and experimental evidence across a number of taxa has begun to support that the formation of novel, or evolution of existing, host-symbiont relationships may facilitate rapid host adaptation and range expansion (e.g., *Sirex* woodwasps: Wooding et al., 2013; Hajek et al., 2013; ants: Mueller et al., 2011; Cheng et al., 2019; mice: Chevalier et al., 2015; pine trees: Gundale et al., 2016; and *Brassica* plants: Lau and Lennon, 2012). Despite these advances, however, assessing causality in the patterns uncovered remains challenging.

In order to more directly investigate the extent to which microbial symbionts themselves facilitate host plasticity and adaptation in the face of environmental change, additional studies are needed where both the microbiome and environmental conditions of a host are directly manipulable. We sought to address this challenge in the bull-headed dung beetle *Onthophagus taurus*, which was introduced from its native Mediterranean range into both Eastern and Western Australia, as well as the Eastern United States (Silva et al., 2016). Introductions into Australia were part of a biocontrol effort to combat dung breeding flies and pasture degradation starting in the late 1960's, required beetles to be surface sterilized as eggs and quarantined for a generation prior to release, followed by extensive re-harvesting and re-distributing in the field to increase the species' introduced range (Edwards, 2007). In contrast, in the Eastern US *O. taurus* appears to have been introduced accidentally from an unknown source population (Vulinec & Eudy, 1993). Since the first documentation of this species in Santa Rosa County, Florida, in 1971, and without the subsequent aid of deliberate redistribution efforts, *O. taurus* managed to spread to Texas in the west, and the Canadian border in the north, ultimately occupying a climatic niche space far exceeding that of both its native Mediterranean and introduced Australian counterparts (Rounds and Floate, 2012; Silva et al., 2016; Floate et al., 2017). However, exactly how EUS *O. taurus* populations were able to disproportionately expand their climatic niche is unclear. Here we test the hypothesis that the expansion of *O. taurus* in the Eastern US was facilitated through local adaptation of beneficial host-microbiome interactions.

Entomologists have long hypothesized that *Onthophagus* beetles are able to feed on their characteristic diet of nutritionally challenging ruminant dung through associations with symbiotic microbes (Goidanich & Malan, 1962; Rougon et al., 1990; Holter, 2016; Frank et al., 2017). As detailed below, recent research increasingly supports this hypothesis. Onthophagine

beetles reproduce via the construction of subterranean brood balls, compact, spherical constructions of dung with an egg chamber containing a single egg within. Work in *O. taurus* has shown that the gut microbial communities of mothers and their larval offspring are highly similar, and that this similarity arises because mothers directly pass their gut microbes to their offspring through a fecal secretion – called the "pedestal" - positioned underneath the egg and consumed by larvae immediately after hatching (Estes et al., 2013). Subsequent work also showed that 1) vertically transmitted pedestal microbes are developmentally important, as *Onthophagus* beetles reared without their pedestals take longer to develop and eclose to smaller adults as compared to conspecifics provided their pedestals during the larval stage (Schwab et al., 2016); 2) these negative growth consequences are exacerbated under stressful environmental conditions but may be rescued through inoculation with pedestal derived bacterial cultures (Schwab et al., 2016); and 3) the microbial communities of *Onthophagus* beetles are diverse and structured both by ancestral associations, and environmental forces which have brought about shifts in microbiome composition in as short as 50 years following the introduction of *O. taurus* into the Eastern US and Australia (Parker et al., 2020). We thus hypothesized that the successful range expansion seen specifically in Eastern US *O. taurus* may be due at least in part to local adaptations in the relationship between beetle hosts and their associated microbiota.

To address this hypothesis, we explored the importance of the pedestal microbiota on developmental outcomes of fitness-related traits including development time, survival rate, and adult body size in two populations of EUS *O. taurus* beetles from Northern Florida (FL) and Northern Michigan (MI) – the southern and northern extremes of the species' current EUS range. Specifically, we assessed: 1) whether beetles derived from these two populations exhibit divergence in the thermal sensitivity of their development, 2) whether both populations show

signatures of local adaptation to thermal conditions by rearing both FL and MI animals at both FL and MI-like soil temperatures; and 3) whether pedestal-derived microbiota facilitate local thermal adaptations by enhancing host fitness in challenging temperature conditions.

MATERIALS AND METHODS

Beetle collection and husbandry

Onthophagus taurus beetles were field collected from two locations in the Eastern United States representing their current southern and northern extremes of their range, and then shipped to Bloomington, IN. In the south, beetles were collected from the UF Santa Fe River Ranch Beef Unit, near Alachua, Florida (29.9242, -82.4950) in early May 2019; and in the north, beetles were collected from the MSU Lake City Research Center, Lake City, Michigan (44.3089, -85.2034) in late August 2019 (Fig. 1). After arriving in the lab, all beetles were transferred into single-population colonies, where they were maintained in a sand/soil mixture at 24ºC and fed antibiotic-free cow dung twice weekly as described in Moczek (2006). Because of differences in collection times between the two populations animals were reared for one generation in the lab before they were used for experiments.

To breed animals for experiments seven adult females and three adult males were allowed to mate and produce brood balls in plastic containers (25cm X 25cm X 13cm) filled with a moist sand/soil mixture and provided dung *ad libitum.* Following protocols described in Parker et al. (2018), brood balls were collected after six days, carefully opened with gloved hands, and eggs inside extracted using autoclave sterilized paintbrushes. Eggs were then surface sterilized with one rinse of 100µL of 1% bleach and 0.1% Triton-X 100 solution, followed by two rinses of 1mL of deionized water. Following this, the maternal fecal deposit unto which the egg was

oviposited (the aforementioned pedestal) was dissected out of the brood ball using a flame sterilized surgical blade. This pedestal was then placed into the center of an artificial brood ball constructed within the well of a twelve-well plate, and a single sterile egg was placed on top all following Parker et al. (2018). Eggs obtained from each population were haphazardly assigned to one of two treatments within each plate: a self-inoculated treatment where each sterilized egg was placed back on its own pedestal, or a cross-inoculated treatment where eggs were placed on a pedestal from the other population. These four resulting treatment groups were blocked vertically within each plate, and their order was randomized to minimize within-plate effects, with three individuals per treatment group in each plate.

Furthermore, each plate was haphazardly assigned to one of two temperature treatments. Plates were stored in an incubator, at either 19ºC or 27ºC for all of development. These temperatures were chosen to mimic peak-breeding season soil temperatures at the MI and FL collection locations, respectively, as obtained from long term monitoring records (from Syngenta, the National Oceanic and Atmospheric Administration, and the US Department of Agriculture National Resources Conservation Service). Plates were then checked once every 48 hours to assess animal growth and stage of development. After each check, the orientation and position of plates within the incubators were changed to further minimize the effects of any potential microclimatic variation within the incubator. Final sample sizes were 30 individuals per treatment at 19ºC and 27 per treatment at 27ºC.

Data collection

To assess the effects of pedestal swapping, and our temperature treatments on the growth, development and survival of our animals we collected the following measurements for each

individual: days until 1) final (third) larval instar, 2) pupation, and 3) adulthood. We also measured the weight of our animals at two timepoints during their development: we first measured larval mass 7 days after an individual was first scored as a third instar. By this time larvae are nearing the peak weight they will obtain during their larval growth period, and we use this measure as an indication of a given larva's ability to maximize mass gain during a 7-day period. We also assessed pupal mass 48h after an individual was scored as a pupa as an estimate of final body mass acquisition after larvae have purged their gut and successfully completed the larval to pupal molt. Pupal mass is typically very closely correlated with adult body size (Moczek, 2006). All mass measurements were recorded to the nearest 0.0001g using a Mettler Toledo AL54 (Mettler, Columbus, Ohio, USA) scientific scale. All animals who reached the pupal stage were sexed to allow for analysis of sex differences in treatment effects. Finally, we also measured time to death for animals that did not survive to adulthood, survival rates, and adult body size (as pronotum width, using a digital camera and ImageJ software as previously described (Moczek, 2006) whenever applicable.

Data analysis

All analyses were performed in R v3.5.3 (R Core Team, 2013) and RStudio (RStudio Team, 2015) using the packages *car* (Fox et al., 2012), *GGally* (Schloerke et al., 2017), *ggplot2* (Wickham, 2016), and *visreg* (Breheny & Burchett, 2017).

To investigate the specific influence of our pedestal manipulations on the various growth, development, and survival metrics measured, we constructed linear mixed, and generalized linear mixed (binomial family error distribution) models regressing these measured variables on all possible main effect combinations, and interactions of pedestal treatment, population, rearing

temperature, and sex. Plate code was included as the random effect in each model to account for random error introduced by our experimental design. The regressors in each model constructed were validated using Wald chi-square tests, and regression diagnostics were performed to check assumptions related to normality of the residuals, homoscedasticity of the variance, and for the presence of outliers or otherwise overly influential points. Non-significant interaction terms were removed, and all higher-order interactions above two-way were never significant. Furthermore, Levene's tests were used to check for equality of variances between measured variables for our different sample groups. The Kaplan-Meier estimator was used to obtain survival curves for each of our eight treatment groups, and the log-rank test was used to compare these curves.

RESULTS

In this study we sought to investigate potential differences in growth, development, and survival between *Onthophagus taurus* beetles across the extremes of their Eastern US range – and to examine to what extent these differences can be attributed to the pedestal microbiome (the primary source of vertical microbial transmission in this genus; Estes et al*.,* 2013; Schwab et al., 2016). To do so we employed a fully factorial experimental design where we manipulated both the rearing temperature (19ºC or 27ºC reflecting peak-breeding season soil temperatures at each location), and pedestal origin (self- or cross-inoculated) of beetles from both Northern Michigan (MI) and North-Central Florida (FL). Our predictions for this experiment were multilayered. First, we expected significant differences in developmental performance metrics between MI and FL populations when reared with their own pedestal (self-inoculated) depending on rearing temperature. Specifically, we expected MI individuals to outperform FL individuals at 19ºC, but

the inverse to manifest at 27ºC. Second, we predicted that our pedestal manipulation would interact with rearing temperature and population to increase fitness in a subset of situations. We found partial support for these predictions.

Population origin affects developmental performance and survival, irrespective of rearing temperature.

FL and MI populations differed significantly in several developmental performance measures and life history traits, consistent with population divergence. At the same time, and contrary to our predictions, we failed to find support for our hypothesis that animals perform better if reared at population-specific rearing temperatures, and that performance differences may be mediated by pedestal-derived microbiota. Specifically, we found that FL larvae and pupae grew to larger sizes and survived at a higher rate compared to MI larvae (Fig. 2; Table 1). These effects were seen in linear mixed and generalized linear mixed models which considered rearing temperature and pedestal treatment in addition to population of origin. In addition to the significant difference seen between MI and FL animals, we observed increased larval and pupal mass, as well as larger adult body sizes and increased survival rates for both populations when reared at 27ºC (Fig. 2; Table 1). In contrast, we saw no significant difference in either larval mass or survival rate between cross- and self-inoculated animals (Table 1).

Despite these differences early on during development, we failed to detect a significant influence of population origin on final adult body size (Table 1). Likewise, even though we saw a significant difference in ultimate survival rate between these two populations there was no significant difference in the slope or shape of their survival curves – as given by the Kaplan-Meier estimator and corresponding log-rank test.

Microbiome swapping across host populations improves developmental performance in both populations, but only at one rearing temperature.

We originally predicted that animals from either population would perform better when reared with their own pedestal microbes. However, we observed precisely the opposite pattern, though only at one of the two rearing temperatures. Larvae derived from both FL and MI populations who received their own pedestal (self-inoculated) developed significantly slower than cross-inoculated larvae (~ 3 days) at 19ºC , but not at 27 ºC (Fig. 3; Table 1**)**. However, as previously noted we saw no significant difference caused by pedestal manipulation in the size of these animals at any life stage (Table 1). That is, in a linear mixed model explaining total development time (egg to adult eclosion) by pedestal treatment, animal population, rearing temperature, and the interaction between rearing temperature and pedestal treatment, the cross-inoculation treatment significantly reduced the time needed to reach adulthood at 19ºC only, but did not affect the size of animals at either of these life-stages. Importantly, population of origin did not affect this pattern as both MI and FL beetles developed faster when subject to the cross-inoculation treatment (Table 1). Furthermore, the interaction between population and pedestal treatment was not significant, meaning cross-inoculation reduced total development time to the same degree in both MI and FL populations at 19ºC. Lastly, we detected no significant differences between male and female individuals for any of the metrics we measured.

DISCUSSION

In this study, we leveraged the rapid range expansion of the bull-headed dung beetle *Onthophagus taurus* in the Eastern US to address whether host-associated microbiota can

mediate local thermal adaptation and host range expansion. We sought to address this question using an experimental design which manipulated both the microbial, and developmental thermal environment of larvae derived from two populations representing the southern and northern extremes of the latitudinal range this species has recently established in the Eastern US (Fig. 1). Below we discuss the most important implications of our results.

Florida-derived beetles outperform Michigan-derived beetles regardless of rearing temperature

Based on earlier studies documenting rapid population differentiation in *O. taurus* (Moczek, 2003; Beckers et al., 2015; Casasa & Moczek, 2018), and the large climatic differences experienced by these beetles over their Eastern US range (Silva et al., 2016), we predicted that populations collected at the southern and northern extremes of this range would show significant divergences in developmental performance and/or life history. We found that in support of these predictions, populations from FL and MI diverged both in adult survival rate and larval size (Fig. 2). At the same time, we were unable to find support for our second prediction that populations would show local adaptation to their respective local thermal conditions as FL-derived beetles outperformed MI-derived beetles regardless of rearing temperature (Fig. 2). This is in contrast to a recent study documenting clinal differentiation and the evolution of genotype-by-environment interactions across Eastern US *O. taurus* populations (Rohner & Moczek, 2020), which, however, assessed four populations, was able to use the offspring of field collected individuals which possibly introduced direct maternal effects that could not be accounted for, and did not require the experimental manipulation of pedestals. Together, these factors might explain the disagreement in findings between these two studies.

Exchanging pedestal microbiota between populations speeds growth at one rearing temperature, consistent with enemy release dynamics

In line with our general predictions, we found that pedestal-microbiome manipulation significantly impacted fitness-related traits in a subset of environmental conditions and genetic backgrounds. However, our specific prediction – that this impact would be fitness enhancing under thermal conditions reflective of the source population – was not met. Instead, we found that providing both MI and FL animals with the other population's pedestal shortened larval development time (yet without affecting final adult body size; Fig. 3; Table 1), a trait directly linked to reduced generation time and increased fitness in many insects (Kingsolver & Huey, 2008). This finding was unexpected because previous research demonstrated that both (i) withholding pedestals (Schwab et al., 2016) and (ii) pedestal swaps across species (Parker et al., 2018) result in negative developmental outcomes, and (iii) that *O. taurus* populations obtained from different exotic ranges – while maintaining a putative core microbiome – also harbor taxonomically distinct microbial communities (Parker et al., 2020). Collectively, this raises the possibility that host-microbiota co-adaptation may not manifest on the level of populations *within* a given range. Instead, our finding that cross-inoculated individuals outperform self-inoculated individuals raises the alternative hypothesis that this enhanced performance occurred because host individuals may have been released from pressures imposed by microbial pathogens while still maintaining a functional core microbiome.

The enemy release hypothesis posits that one reason why non-native species often outperform their native counterparts is that they have been released from the pressures imposed by natural enemies (such as parasites, predators, or microbial pathogens) in their native range (Mitchell et al., 2006; Reinhart & Callaway, 2006). While most commonly invoked in plant

systems, this hypothesis is equally applicable to animal systems – and in fact patterns consistent with this hypothesis have been observed in a number of animal taxa (Torchin et al., 2003; Marr et al., 2008; Ross et al., 2010). Furthermore, growing evidence highlights the context-dependent nature of host-microbe relationships. Microbial symbionts can evolve mutualistic relationships with their hosts under certain contexts, but as those conditions change – i.e. if a pathogen does not occur in a newly colonized host environment, or if a host's diet changes – these relationships can shift and become neutral or even deleterious to host fitness (Gerardo & Parker, 2014; Corbin et al., 2017). Our results are consistent with a scenario whereby pedestal microbiota exchange between MI and FL *O. taurus* populations resulted in a release from negative pressures which in turn lead to accelerated host development (Kingsolver & Huey, 2008). If correct, these findings raise the possibility that host range expansions as seen in *O. taurus* may be facilitated not only by the acquisition of beneficial microbial interactions, but also by the location-specific removal of *negative* microbial challenges. Future studies comparing pathogen loads of various *O. taurus* populations from both their native Mediterranean and exotic Eastern US ranges would help to directly test this hypothesis.

Finally, it is worth noting that microbiome swapping enhanced larval development of both populations, yet at only one temperature, the Michigan like 19°C, but not the 27°C meant to reflect Florida soil temperatures. This suggests that the interactions between host and microbial physiology that influence development time and growth, whatever those may be, are themselves temperature sensitive. This may not be that surprising, however, because on one side a robust body of work has already demonstrated the temperature dependence of fitness relevant traits in *Onthophagus* (e.g. development time, size at pupation, eclosion success; Floate et al., 2014; Macagno et al., 2016; Macagno et al., 2018; Rohner et al., 2020), while on the other diverse

aspects of the external environment, including temperature, are well known to impact host microbiome interactions in other systems (Renoz et al., 2019). Combined, our results thus raise the possibility that the relatively slow host metabolism and growth possible at 19°C may allow population-specific microbiome members to exert their growth limiting effects, whereas the more rapid host metabolism and growth possible at 27°C may override the influences of individual microbiome members regardless of their specific origin, hypotheses that clearly warrant further scrutiny.

CONCLUSION

Understanding how animals respond to environmental conditions is of the utmost importance in a rapidly changing world. The role and significance of host-associated microbiota in this context remains understudied (Sudakaran et al., 2017). Our results provide an example of the complex ways in which changes in host-microbiota associations may limit or facilitate successful range expansions. Specifically, our work raises the possibility that successful range expansions in dung beetles, rather than being facilitated through the acquisition of beneficial microbial interactions may in addition, or instead, be enabled by the release from *negative* microbial challenges. Though more work is clearly needed to assess this particular hypothesis, our results underscore how host-microbiome interactions may complicate host responses to environmental change.

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REFERENCES

Barrett, R. D., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in ecology & evolution*, *23*(1), 38-44.

Beckers, O. M., Anderson, W., & Moczek, A. P. (2015). A combination of developmental plasticity, parental effects, and genetic differentiation mediates divergences in life history traits between dung beetle populations. *Evolution & development*, *17*(2), 148-159.

Breheny, P. and Burchett, W. (2017) Visualization of Regression Models Using visreg. *The R Journal*, 9: 56-71.

Casasa, S., & Moczek, A. P. (2018). The role of ancestral phenotypic plasticity in evolutionary diversification: population density effects in horned beetles. *Animal Behaviour*, *137*, 53-61.

Cheng, D., Chen, S., Huang, Y., Pierce, N. E., Id, M. R., Yang, F., … Id, Y. X. (2019). Symbiotic microbiota may reflect host adaptation by resident to invasive ant species. *PLoS Pathogens*, 1–22.

Chevalier, C., Stojanović, O., Colin, D. J., Suarez-Zamorano, N., Tarallo, V., Veyrat-Durebex, C., … Trajkovski, M. (2015). Gut Microbiota Orchestrates Energy Homeostasis during Cold. *Cell*, *163*(6), 1360–1374.

Corbin, C., Heyworth, E. R., Ferrari, J., & Hurst, G. D. D. (2017). Heritable symbionts in a world of varying temperature. *Heredity*, *118*(1), 10–20.

Edwards, P. (2007) Introduced Dung Beetles in Australia 1967-2007, 1–66.

Estes, A. M., Hearn, D. J., Snell-Rood, E. C., Feindler, M., Feeser, K., Abebe, T., … Moczek, A. P. (2013). Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS ONE*, *8*(11), 1–15.

Floate, K. D., Watson, D. W., Coghlin, P., & Olfert, O. (2014). Degree-day models for development of the dung beetles *Onthophagus nuchicornis*, *O. taurus*, and *Digitonthophagus gazella* (Coleoptera: Scarabaeidae), and the likelihood of *O. taurus* establishment in southern Alberta, Canada. *Canadian Entomologist*, *147*(5), 617–627.

Floate, K. D., Watson, D. W., Weiss, R. M., & Olfert, O. (2017). Bioclimatic analyses for the distributions of *Onthophagus nuchicornis*, *Onthophagus taurus*, and *Digitonthophagus gazella* (Coleoptera: Scarabaeidae) in North America. *Canadian Entomologist*, *149*(4), 504–524.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., *et al.* (2012) Package 'car'. Vienna: *R Foundation for Statistical Computing*.

Frank, K., Brückner, A., Hilpert, A., Heethoff, M., & Blüthgen, N. (2017). Nutrient quality of vertebrate dung as a diet for dung beetles. *Scientific Reports*, *7*(1), 1–12.

Gerardo, N. M., & Parker, B. J. (2014). Mechanisms of symbiont-conferred protection against natural enemies: An ecological and evolutionary framework. *Current Opinion in Insect Science*, *4*(1), 8–14.

Goidanich, A., & Malan, C. E. (1962). *Sulla fonte di alimentazione e sulla microflora aerobica del nido pedotrofico e dell'apparato digerente delle larve di scarabei coprogagi:(Coleoptera scarabaeidae)*.

Gundale, M. J., Almeida, J. P., Wallander, H., Wardle, D. A., Kardol, P., Nilsson, M. C., … Austin, A. (2016). Differences in endophyte communities of introduced trees depend on the phylogenetic relatedness of the receiving forest. *Journal of Ecology*, *104*(5), 1219–1232.

Hajek, A. E., Nielsen, C., Kepler, R. M., Long, S. J., & Castrillo, L. (2013). Fidelity Among *Sirex* Woodwasps and Their Fungal Symbionts. *Microbial Ecology*, *65*(3), 753–762.

Holter, P. (2016). Herbivore dung as food for dung beetles: elementary coprology for entomologists. *Ecological Entomology*, *41*(4), 367–377.

Kingsolver, J. G., & Buckley, L. B. (2017). Evolution of plasticity and adaptive responses to climate change along climate gradients. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1860), 2–8.

Kingsolver, J. G., & Huey, R. B. (2008). Size, temperature, and fitness: three rules. *Evolutionary Ecology Research*, *10*(2), 251-268.

Macagno, A. L., Moczek, A. P., & Pizzo, A. (2016). Rapid divergence of nesting depth and digging appendages among tunneling dung beetle populations and species. *The American Naturalist*, *187*(5), E143-E151.

Macagno, A. L., Zattara, E. E., Ezeakudo, O., Moczek, A. P., & Ledón-Rettig, C. C. (2018). Adaptive maternal behavioral plasticity and developmental programming mitigate the transgenerational effects of temperature in dung beetles. *Oikos*, *127*(9), 1319-1329.

Marr, S. R., Mautz, W. J., & Hara, A. H. (2008). Parasite loss and introduced species: a comparison of the parasites of the Puerto Rican tree frog,(Eleutherodactylus coqui), in its native and introduced ranges. *Biological Invasions*, *10*(8), 1289-1298.

Merilä, J., & Hendry, A. P. (2014). Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. *Evolutionary applications*, *7*(1), 1-14.

Mitchell, C. E., Agrawal, A. A., Bever, J. D., Gilbert, G. S., Hufbauer, R. A., Klironomos, J. N., ... & Seabloom, E. W. (2006). Biotic interactions and plant invasions. *Ecology letters*, *9*(6), 726-740.

Moczek, A. P. (2003). The behavioral ecology of threshold evolution in a polyphenic beetle. *Behavioral Ecology*, *14*(6), 841-854.

Moczek, A. P. (2006) Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. The American Naturalist, 168(6), 711-729.

Mooney, H., Larigauderie, A., Cesario, M., Elmquist, T., Hoegh-Guldberg, O., Lavorel, S., … Yahara, T. (2009). Biodiversity, climate change, and ecosystem services. *Current Opinion in Environmental Sustainability*, *1*(1), 46–54.

Mueller, U. G., Mikheyev, A. S., Hong, E., Sen, R., Warren, D. L., Solomon, S. E., … Juenger, T. E. (2011). Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant-fungus symbiosis. *Proceedings of the National Academy of Sciences*, *108*(10), 4053–4056.

Parker, E. S., Dury, G. J., & Moczek, A. P. (2019). Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles. *Ecological Entomology*.

Parker, E. S., Newton, I. L. G., & Moczek, A. P. (2020). (My Microbiome) Would Walk 10,000 miles: Maintenance and Turnover of Microbial Communities in Introduced Dung Beetles. *Microbial Ecology*.

R Core Team (2013). R: A language and environment for statistical computing.

Reinhart, K. O., & Callaway, R. M. (2006). Soil biota and invasive plants. *New phytologist*, *170*(3), 445-457.

Renoz, F., Pons, I., & Hance, T. (2019). Evolutionary responses of mutualistic insect–bacterial symbioses in a world of fluctuating temperatures. *Current Opinion in Insect Science*, *35*, 20–26.

RStudio Team (2015) RStudio: integrated development for R. RStudio, Inc., Boston, MA

Rohner, P., & Moczek, A. P. (2020). Rapid differentiation of plasticity in life history and morphology during invasive range expansion and concurrent local adaptation in the horned beetle *Onthophagus taurus*. *Evolution*.

Ross, J. L., Ivanova, E. S., Severns, P. M., & Wilson, M. J. (2010). The role of parasite release in invasion of the USA by European slugs. *Biological Invasions*, *12*(3), 603-610.

Rougon, D., Rougon, C., Levieux, J., & Trichet, J. (1990). Variations in the amino-acid content in zebu dung in the Sahel during nesting by dung-beetles (Coleoptera, Scarabaeidae). *Soil Biology and Biochemistry*, *22*(2), 217–223.

Rounds, R. J., & Floate, K. D. (2012). Diversity and Seasonal Phenology of Coprophagous Beetles at Lake City, Michigan, USA, with a New State Record for *Onthophagus taurus* (Schreber) (Coleoptera: Scarabaeidae). *The Coleopterists Bulletin*, *66*(2), 169–172.

Schloerke, B., Crowley, J., Cook, D., Briatte, F., Marbach, M., Thoen, E., ... & Larmarange, J. (2017). GGally: Extension to 'ggplot2'(R Package Version 1.3.1).

Schwab, D. B., Riggs, H. E., Newton, I. L. G., & Moczek, A. P. (2016). Developmental and Ecological Benefits of the Maternally Transmitted Microbiota in a Dung Beetle. *The American Naturalist*, *188*(6), 000–000.

Silva, D. P., Vilela, B., Buzatto, B. A., Moczek, A. P., & Hortal, J. (2016). Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, 18(11), 3137–3148.

Sudakaran, S., Kost, C., & Kaltenpoth, M. (2017). Symbiont Acquisition and Replacement as a Source of Ecological Innovation. *Trends in Microbiology*.

Torchin, M. E., Lafferty, K. D., Dobson, A. P., McKenzie, V. J., & Kuris, A. M. (2003). Introduced species and their missing parasites. *Nature*, *421*(6923), 628-630.

Vulinec, K., & Eudy, S. P. (1993). A southern range extension for the introduced dung beetle *Onthophagus taurus* Schreber (Coleoptera: Scarabaeidae). *Coleopterists Bulletin*, *47(2)*(January 1993), 129–130.

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Wickham, H. (2016). ggplot2: elegant graphics for data analysis. Springer.

Wooding, A. L., Wingfield, M. J., Hurley, B. P., Garnas, J. R., De Groot, P., & Slippers, B. (2013). Lack of fidelity revealed in an insect-fungal mutualism after invasion. *Biology Letters*, *9*(4).

FIGURES AND LEGENDS

Fig. 1: Collection sites and experimental design. (a) Field collection sites used for this study. Santa Fe, FL and Lake City, MI mark the southern and northern extremes of the *Onthophagus taurus* distribution in the Eastern United States, respectively. **(b)**
Diagram of experimental procedure. F1 animals were used to generate eggs for experimental manipulation. F2 eggs were assigned to either their own pedestal (self-) or pedestals derived from the other population (cross-inoculated). Animals from all four experimental groups were then reared at either 19ºC (MI conditions) or 27ºC (FL conditions).

A


Lake City, MI
Santa Fe, FL

B

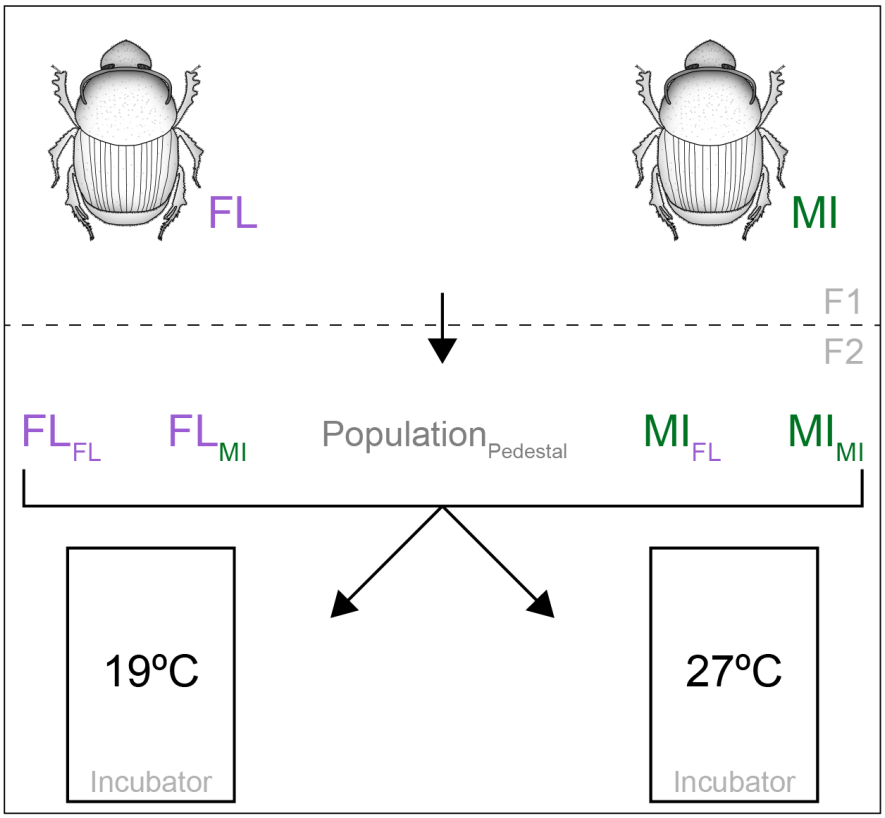
FL
MI
F1
F2
FL FL
FL MI
Population Pedestal
MI FL
MI MI
19ºC
Incubator
27ºC
Incubator

Fig. 2: Effect of population of origin and rearing temperature on development and survival. Effect plots showing the estimated influence of population of origin, and rearing temperature on A) weight at day 7 of the final larval instar (n = 151), B) weight at day 2 of the pupal stage (n = 125) and C) probability of death before adulthood for all animals (n = 228). All plots were derived from either linear mixed (A and B) or generalized linear mixed (C) models containing the factors rearing temperature, population of origin, pedestal treatment, and random factor of plate code. Animals from the FL population (regardless of temperature), and animals reared at higher temperatures (regardless of population) showed higher fitness for both measured variables. Points (A and B) indicate partial residuals, vertical dashes (rug plots in C) indicate individual datapoints in each group, and horizontal colored lines indicate predicted value in each plot.

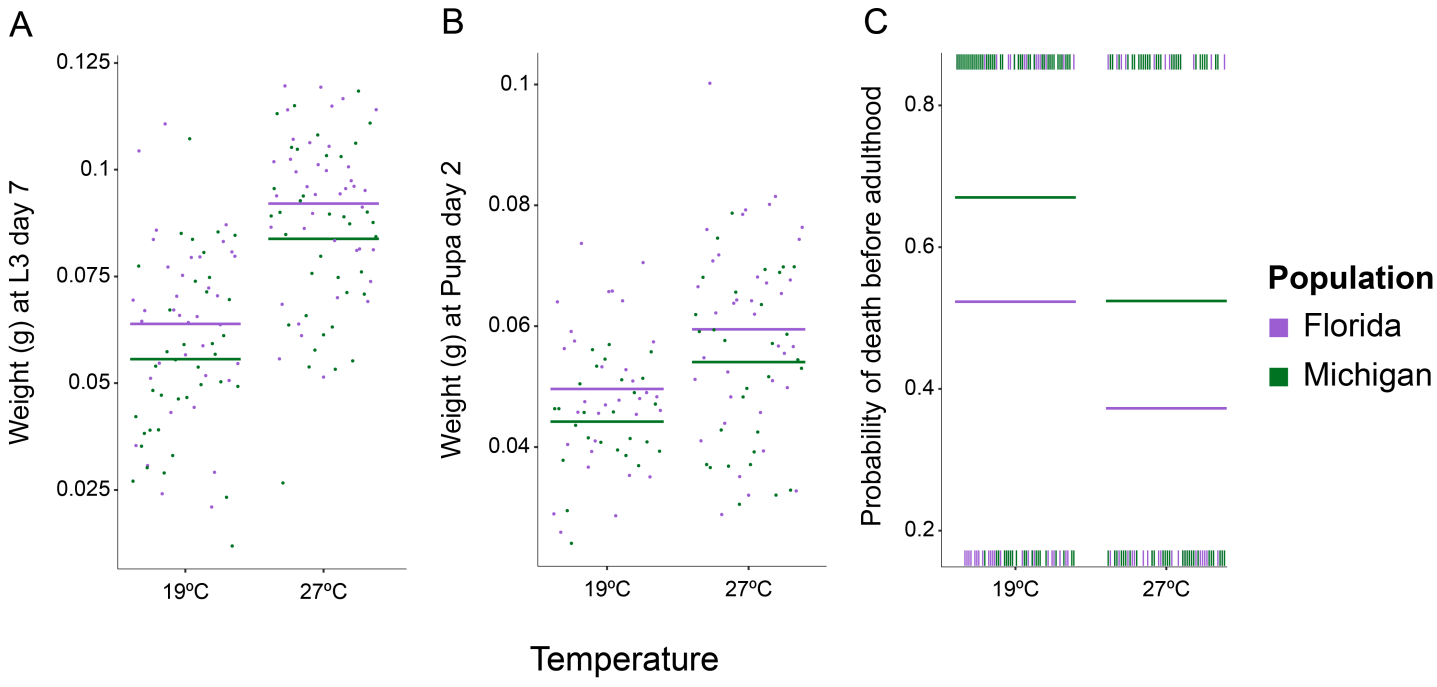

Fig. 3: Effects of pedestal manipulation. Effect plot showing the estimated influence of pedestal manipulation on days until adult eclosion (n = 111). Generated from a linear mixed model containing the factors rearing temperature, pedestal treatment, population of origin, the random factor plate code, and the interaction between pedestal treatment and rearing temperature. Animals which received the other population's pedestal (Cross-inoculated) reached adulthood faster than animals which received their own pedestal (Self-inoculated), but only at 19ºC. Points indicate partial residuals, and colored lines indicate predicted value. Diagonal, dotted lines added to help denote the significant interaction between temperature and pedestal treatment as visualized by the difference in slopes between treatment groups.

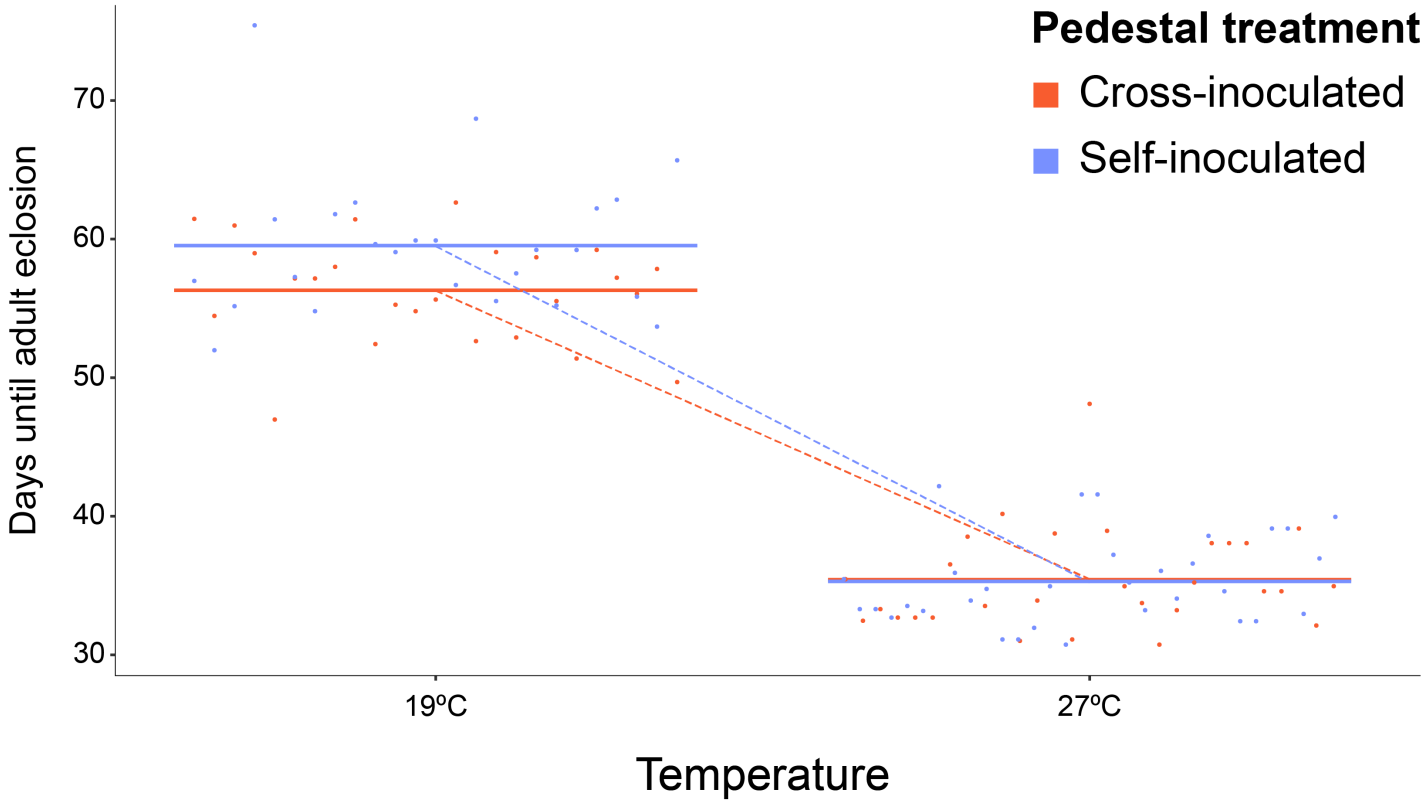

Table 1: Mixed models testing for the significance of rearing temperature, population of origin, and pedestal treatment, on fitness-related developmental metrics. 12-well plate code was used as a random effect in each model. Non-significant interactions were removed, and

three-way interactions were never significant. Columns show Chi-squared test statistic values, resulting test probabilities, and estimated effect sizes and standard error for each regression term.

	larval weight			pupal weight			adult size			survival			total development time		
	X^2	p	$\beta \pm SE$ (g)	X^2	p	$\beta \pm SE$ *(g)*	X^2	p	$\beta \pm SE$ *(mm)*	X^2	p	$\beta \pm SE$ *(prob.)*	X^2	p	$\beta \pm SE$ *(days)*
population	5.86	**0.015**	-0.0082±0.0034	5.643	**0.018**	-0.0054±0.0025	1.101	0.294	-0.076±0.073	5.135	**0.023**	0.65±.27	1.067	0.302	-0.84±0.81
pedestal	1.22	0.269	-0.0038±0.0034	0.256	0.614	-0.00037±0.0029	0.076	0.783	0.02±0.072	0.166	0.684	0.47±.27	3.039	0.081	3.23±1.19
temperature	28.769	**<.001**	0.028±0.0053	11.698	**<.001**	0.0099±0.0033	14.512	**<.001**	0.29±0.076	4.952	**0.026**	0.35±.28	412.219	**<.001**	-20.88±1.37
temperature X pedestal													4.36	**0.037**	-3.35±1.61

CHAPTER 5

(My microbiome) would walk 10,000 miles: Maintenance and turnover of microbial communities in introduced dung beetles.

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ABSTRACT

Host-associated microbes facilitate diverse biotic and abiotic interactions between hosts and their environments. Experimental alterations of host-associated microbial communities frequently decrease host fitness, yet much less is known about if and how host-microbiome interactions are altered by natural perturbations, such as introduction events. Here, we begin to assess this question in *Onthophagus* dung beetles, a species-rich and geographically widely distributed genus whose members rely on vertically transmitted microbiota to support normal development. Specifically, we investigated to what extent microbiome community membership shifts during host introduction events and the relative significance of ancestral associations and novel environmental conditions in the structuring of microbial communities of introduced host species. Our results demonstrate that both evolutionary history and local environmental forces structure the microbial communities of these animals, but that their relative importance is shaped by the specific circumstances that characterize individual introduction events. Furthermore, we identify

microbial taxa such as *Dysgonomonas* that may constitute members of the core *Onthophagus* microbiome regardless of host population or species, but also *Wolbachia* which associates with *Onthophagus* beetles in a species or even population-specific manner. We discuss the implications of our results for our understanding of the evolutionary ecology of symbiosis in dung beetles and beyond.

INTRODUCTION

During ontogeny all animals face the challenge of contending with and responding to a diverse array of environmental influences. For example, host-associated microbes play important roles in the instruction of host development (e.g. nematodes: Foray et al., 2018; mice: Hooper & Gordon, 2001; Sommer & Bäckhed, 2013; and cephalopods: McFall-Ngai, 2014), life-history traits and timing (like metamorphosis induction in marine invertebrates: Shikuma et al., 2014; Sneed et al., 2014; Whalan & Webster, 2014; reproductive timing in plants: Leonardo & Mondor, 2006; and survival-reproduction trade-offs in invertebrates: Emelianoff et al., 2008), learning (Vuong et al., 2017; Chu et al., 2019), and nutritional supplementation in a variety of taxa (Douglas, 2009; Gilbert, 2019). In these and many other contexts experimental alterations of host-associated microbial communities decrease host fitness and result in pathologies (McFall-Ngai et al., 2013; Rosenberg & Zilber-Rosenberg, 2013; Sommer & Bäckhed, 2013; Morimoto et al., 2017). Yet much less is known about if and how host-microbiome interactions are altered during natural perturbations, for example when hosts colonize new geographic regions or habitats. Here, we begin to assess this question in *Onthophagus* beetles which have previously been shown to rely on a vertically transmitted microbiome throughout their development (Schwab et al., 2016; Parker et al., 2018). Specifically, we ask to what extent microbiome community membership

shifts during host introduction events and the relative significance of ancestral associations and novel environmental conditions in the structuring of microbial communities of introduced host species.

Onthophagus dung beetles are one of the most species-rich genera of animals, with over 2,000 described species (Tarasov & Kabakov, 2010). Yet this great species richness has arisen seemingly despite the inherent challenges dung beetles face in consuming dung as their sole food source throughout all stages of their life. Dung, particularly the ruminant dung on which the vast majority of *Onthophagus* species feed, is a nutritionally challenging food source deficient in amino acids and comprised primarily of recalcitrant carbon sources such as cellulose and lignin (Holter, 2016; Frank et al., 2017). Dung beetles have thus long been hypothesized to meet these dietary challenges through association with symbiotic microorganisms (Goidanich & Malan, 1962; Rougon et al., 1990), and recent findings have begun to provide experimental support for this prediction. Work in *O. taurus* and the closely related genus *Euoniticellus* has demonstrated that the gut microbial communities of mothers and their offspring are highly similar, but distinct from the dung they feed on and the soil they live in; and that these gut microbes are reliably passed from mother to offspring through a "pedestal" – a fecal secretion onto which mothers oviposit their eggs (Estes et al., 2013; "maternal gift" in Shukla et al., 2016). Shortly after hatching, larvae consume the pedestal before continuing on to feed upon the remainder of the brood ball provisioned for them by their mother. Parallel work has further demonstrated that the microbes found within the pedestal are functionally significant, as 1) *Onthophagus* beetles reared without their pedestal microbiota are slower to develop and eclose to smaller adults compared to individuals given access to their pedestals as larvae (Schwab et al., 2016); 2) the negative growth consequences of pedestal-free development can be erased by re-inoculating larvae with pedestal

derived bacteria cultivated in the laboratory (Schwab et al., 2016); and 3) the developmental benefits conferred by pedestals are host species-specific, i.e. *Onthophagus* beetles provided another species' pedestal during the egg stage suffer negative effects to their survival, and development – a subset of which continue to persist into the next generation (Parker et al., 2018). Taken together, a growing body of evidence thus supports the notion that *Onthophagus* beetles engage in mutualistic and at least partly host-specific interactions with vertically transmitted gut microbiota.

At the same time, *Onthophagus* dung beetles present an excellent model to understand how host introductions may influence host associated microbial communities. Diverse *Onthophagus* species have been subjected to recent introductions outside their native range as a result of both accidental releases as well as biocontrol programs intended to curb dung accumulation and the corresponding nuisance fly populations on pastureland. For example, *O. taurus* is native to the Mediterranean but became introduced into both Western and Eastern Australia as part of a biocontrol effort starting in the late 1960s (Edwards, 2007; Silva et al., 2016). These introductions entailed a rigorous quarantine procedure which included the surface sterilizing of eggs and their subsequent rearing in artificial brood balls to exclude the possibility of co-introducing potentially harmful microorganisms as well as nematodes and mites commonly associated with dung beetles (Edwards, 2007). Upon introduction, exotic populations where then subject to repeated harvest and redistribution efforts to aid in their further range-wide establishment. In contrast, *O. taurus* was introduced into the Eastern United States by accident around 1971 from an unknown source population (Vulinec & Eudy, 1993). From its origination in a single location in Northern Florida, this population has since expanded as far west as Texas, and as far north as the Canadian border (Silva et al., 2016; Floate et al., 2017) yet did so without

the aid of deliberate redistribution efforts by people. These repeated introductions, coupled with the divergent circumstances surrounding them, therefore make *O. taurus* a promising candidate species to investigate the impact of introduction events on microbiome composition.

In this study we sought to compare and contrast the microbiome of *Onthophagus taurus* from native Mediterranean (MED) as well as exotic Eastern United States (EUS) and Eastern Australia (EA) ranges. Furthermore, we characterized the microbiota of three additional dung beetle species (*O. hecate, O. australis, Euoniticellus fulvus*) native to and syntopic (i.e. often occurring within the same dung pad) with *O. taurus* in each region to allow us to begin assessing the relative contributions of evolutionary history and local forces in driving microbiome assembly. Specifically, we aimed to test two hypotheses: 1) If dung beetle microbiota are structured primarily by evolutionary history, *O. taurus* microbial communities should remain similar regardless of region, and distinct from those of resident native species. 2) Alternatively, if dung beetle microbiota assembly is structured primarily by environmental factors, *O. taurus* microbial communities should be largely distinct between regions and instead more closely resemble the communities of respective native host species.

MATERIALS AND METHODS

Sample collection

Onthophagus taurus and native, sympatric, beetles were field collected from three different geographic regions and shipped to Bloomington, IN. In each region, beetle species pairs were collected on cow dung produced by cattle grazing on pastures subject to a temperate to Mediterranean-type climate. Specifically, *O. taurus* and *Euoniticellus fulvus* (final $n = 8$ each) representing the Mediterranean region (MED) were collected near Turin, Italy, while in the

Eastern United States (EUS) *O. taurus* and *O. hecate* (*n* = 3 each) were collected near Morgantown, WV. Beetle abundances in this region were consistently low during the collection period, leading to a lower sample size for species collected from this region. Lastly, Eastern Australian (EA) *O. taurus* and *O. australis* (*n* = 8 each) were collected near Canberra, Australia. Immediately after arrival all beetles were flash frozen in liquid nitrogen, and then stored at -80C until used for nucleic acid extraction.

RNA Extraction and Amplicon Library Preparation

We chose to analyze RNA for this study as it provides information about bacterial taxa that were alive and likely metabolically active at the time host beetles were frozen (De Vrieze et al., 2016). Before extraction of RNA from each sample, animals were first surface sterilized with 100µL of 1% bleach and 0.1% Triton-X 100 solution followed by two rinses of 1mL of deionized water. Samples were then ground in liquid nitrogen using a previously autoclaved, ceramic mortar and pestle washed with RNase away solution (Molecular BioProducts, San Diego, California, USA). RNA was extracted from each sample using a modified RNeasy PowerSoil total RNA kit (Qiagen, Hilden, Germany) protocol after which residual genomic DNA contamination was subsequently removed using a DNase max kit (Qiagen). The quality and quantity of the cleaned, total RNA was then determined with a TapeStation 2200 (Agilent, Santa Clara, California, USA). Samples determined to be of good quality were then converted to cDNA following the iScript cDNA synthesis kit (BioRad, Hercules, California, USA) protocol.

Amplicon libraries of the V4 region of the 16S SSU rRNA were generated following the Earth Microbiome protocol (515F-806R primers; Caporaso et al., 2012), with some differences. HF Phusion polymerase mix (New England BioLabs, Ipswich, Massachusetts, USA) and 3%

dimethylsulfoxide (DMSO) were used in PCR reactions which were cycled at 98°C for 45 s, 60°C for 60 s, and 72°C for 90 s repeated 35 times in a Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Each sample was amplified in triplicate and then pooled before normalization based on concentration of DNA measured with Qubit 4 fluorometer (ThermoFisher, Waltham, Massachusetts). Final amplicon pool was cleaned following the standard QIAquick PCR purification kit (Qiagen) protocol before being sent to the Indiana University Center for Genomics and Bioinformatics (Bloomington, Indiana, USA) for sequencing.

Amplicon Sequencing and Processing

Pooled amplicons were sequenced using an Illumina MiSeq and 250bp paired-end chemistry (Illumina, San Diego, California, USA). Raw reads with primers and adapters removed were then processed using the software suite mothur v1.42.1 (Schloss et al., 2009). First, contigs were generated using the make.contigs() command. Sequences were then trimmed for length and ambiguous base pairs were removed using screen.seqs(maxambig = 0, maxlength = 275). Unique sequences were then aligned to v132 of the SILVA 16S reference alignment (Quast, 2012), trimmed to overlap only homologous regions, and preclustered based on a nucleotide difference of two. Chimeric sequences were identified and removed using the chimera.vsearch() command. OTUs identified as potential contaminants in the blank (all belonging to the Acinetobacter, Enterococcaceae_unclassified, Bacillales_unclassified, or Dysgonomonas lineages) were removed from all samples. Additionally, one sample (EA *australis* 2.1.19.2) identified as a likely sick animal, and all sequences classified as chloroplast, mitochondria, Archaea, Eukaryota, or unknown, were removed from the dataset. Retained sequences were classified using a custom

training set based on SILVA v132 at a confidence threshold of 80, and then clustered into OTUs (operational taxonomic units) with a 97% identity threshold. To generate the primary dataset used for our analyses, we then further removed all sequences classified as *Wolbachia* as this genus was so common in some samples (up to 71% of all reads) that it made analysis of other, less common, taxa difficult. All samples in this primary dataset were rarefied to about 23,000 sequences, roughly the size of the smallest sample. Finally, the get.oturep() command was used to obtain a representative DNA sequence for each OTU after which FastTree v2.1.10 (Price et al., 2010) was used to generate an approximately-maximum-likelihood phylogeny using the GTR-CAT model.

Data Analysis

Analysis of the final dataset was performed in R v3.5.3 (R Core Team, 2013) using the packages phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2018), and ggplot2 (Wickham, 2016). Various alpha diversity estimates (Chao1, Shannon Index, Simpson Index) and between-sample distances (Bray-Curtis, unweighted UniFrac, weighted UniFrac) were computed. Distance matrices were then used to cluster samples using Non-metric MultiDimensional Scaling (NMDS). Two-way ANOVA tests of the alpha diversity estimates, and PERMANOVAs and ANOSIMs on the distance matrices were used to test for statistically significant differences in microbiota composition and diversity between sample groups. Assumptions of ANOVA (normality and homoscedasticity) were validated visually (with Q-Q plots) and statistically (using the Levene's test for equality of variance). Further statistical analyses of differentially abundant OTUs was performed using the mothur implementation of the Metastats program (White et al., 2009).

RESULTS

Our 250bp paired end MiSeq run resulted in a total of 15,430,451 reads. Of these, 23%, or 3,604,277 reads, passed all quality control and cleaning steps (including the removal of highly prevalent *Wolbachia* reads), resulting in a final range of 22,646 to 173,634 reads per sample in our primary dataset. The dataset was then rarefied to the size of the smallest sample (22,646 reads). 7,109 bacterial OTUs were identified in the rarefied dataset at 97% identity. Rarefaction curves (Fig. S1) show that our chosen rarefaction cutoff point captures the vast majority of microbial diversity in most samples. This conclusion was supported by estimates of Good's coverage (1 - (number of individuals in species / total number of individuals)) which ranged from 97.8-99.8% for all samples in the rarefied dataset. To facilitate comparisons across individuals and taxa we generated a filtered dataset with representing common OTUs – defined as those found in at least one sample, at at least 5% total relative abundance. These criteria identified 42 common OTUs (Fig. 2), 41 of which were classified to four bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria), and one which was unclassified at the phylum level.

Alpha and Beta Diversity

The most abundant bacterial phylum in our primary dataset was Proteobacteria with an average abundance of 45.5% (15.4-75.3% per sample), followed by Bacteroidetes (31.3% average, 9.5-68.6% per sample), Firmicutes (15.6%, 1.02-52.1%), and Actinobacteria (5.9%, 0.327-20.9%). Reads which were unclassified, or belonged to other, rarer, phyla accounted for the remaining 1.7%. To investigate differences in alpha diversity between samples, we calculated estimates for

the Chao1, Shannon, and Simpson diversity indices (Fig. S2). We did not detect any significant differences in Shannon and Simpson alpha diversity metrics between sample groups (ANOVA, $p = 0.575$ and $p = 0.45$ respectively). In contrast, Chao1 diversity estimates were significantly different between sample groups (ANOVA, $p = 0.00792$), however, this result may have been influenced by the unusually large estimated microbial diversity of the three EUS *O. taurus*. Consistent with this interpretation, the removal of these three samples from the dataset brought the Chao1 test results in line with the other two (ANOVA, $p = 0.426$). Furthermore, no statistically significant difference was found in the within-sample group variation for any of the alpha diversity estimates for either the full (Levene's test: Shannon, $p = 0.4539$; Simpson, 0.8172; Chao1, 0.6617) or *O. taurus* only (Levene's test: Shannon, $p = 0.2671$; Simpson, 0.1658; Chao1, 0.7301) datasets.

To investigate potential differences in microbial community membership between sample groups we performed permutational multivariate analysis of variance (PERMANOVA), analysis of similarities (ANOSIM), clustering analysis, and ordination using non-metric multidimensional scaling (NMDS). Microbial communities of samples tested were found to be significantly different based on both the beetle species they were extracted from (weighted UNIFRAC, PERMANOVA; $p < 0.001$), and the region a given beetle originated from ($p < 0.001$). Specifically, in a model that considers sequentially the region from which a given beetle was obtained and a given beetle' species identity the amount of variance explained by these two factors was 16.7% and 26% respectively (weighted UNIFRAC; both factors were significant and explained similar amounts of variance when unweighted UNIFRAC, and Bray-Curtis distances were considered). Additionally, microbial communities largely clustered in a manner that reflected both host species identity, and region – save for *O. taurus* from Eastern Australia (Fig.

3). While the microbiomes of the Mediterranean samples clustered tightly both within and between species (weighted UniFrac, ANOSIM; $R = 0.687$, $p < 0.001$), those of *O. taurus* introduced to Eastern Australia were split between clustering primarily with the Mediterranean group (weighted UniFrac, ANOSIM; $R = 0.646$, $p < 0.001$), and the native Australian representative species, *O. australis* (weighted UniFrac, ANOSIM; $R = 0.307$, $p = 0.006$). *Onthophagus australis* showed a similar but less pronounced spread between samples, and clustered largely separately from the other groups and most closely to Australian *O. taurus* (weighted UniFrac, ANOSIM; $R = 0.307$, $p = 0.005$), likely due to the large within sample variance seen in each group.

Common OTUs Associated with Dung Beetles

Of the 42 most common OTUs in our primary dataset (which excluded OTUs identified as contaminants and *Wolbachia*), two (OTUs 16, and 55) were classified as *Apibacter*. Reads from these OTUs were found in high abundance primarily in non-*taurus* samples collected in the Mediterranean and Eastern Australia (Fig. 2, Fig. S3). In fact, these OTUs were either completely absent from or at exceedingly low abundances in all *Onthophagus taurus* samples analyzed. Indeed, OTU 16 was found to be significantly more abundant in MED *E. fulvus* compared to MED *O. taurus* (using Metastats, average abundance of 12.3 vs <0.01%, $p < 0.001$). All beetle species examined, across all three regions, were found to be associated with OTUs classified as *Dysgonomonas*. Five different OTUs (8, 20, 32, 45, and 56) were found in the common dataset, with multiple OTUs often appearing in the same sample. In the larger, rarefied, dataset 855 OTUs in total were classified to the genus *Dysgonomonas* – roughly 12% of all OTUs identified. Further, four OTUs (5, 9, 15, and 37) classified as “bacterium endosymbiont of

Onthophagus taurus" using a training set with data from a previous study of the *O. taurus* microbiome (Estes et al., 2013). Two of these, OTUs 9 and 15, fell within the genus *Pasteurella* while the other two, 5 and 37, were classified as *Pseudomonas* and *Desulfovibrio,* respectively. While the study which originally identified these bacteria was performed on *O. taurus* beetles only, our data suggest that these taxa may contribute to the microbiota of multiple dung beetle species. This appears particularly true for *Pseudomonas* which was seen at high abundance in nearly every sample, and also for *Pasteurella* which was common in three species: Mediterranean *O. taurus* and *Euoniticellus fulvus*, and Eastern Australian *O. australis*. However, a subset of these OTUs did exhibit marked differences in relative abundances across samples: for example, *Pasteurella* OTU 9 was significantly more common in the native, source MED *O. taurus* population as compared to the introduced EA *O. taurus* (7.3 vs 0.047% average abundance, $p < 0.001$), while the other common *Pasteurella* OTU (15) was found almost exclusively in EA *O. australis* but rare in any other sample group including EA *O. taurus* (9% vs < 0.01% average abundance, $p < 0.001$)

Finally, one OTU (4) classified as Weeksellaceae showed a largely *O. taurus* biased association. This OTU was present in all *O. taurus* samples, often at high relative abundance (average of 15%, ranging from 0.02 to 42.5%) though it was largely absent from samples of other species. This observation of differential abundance was further supported by the results of the Metastats program which showed that OTU 4 was significantly enriched in Mediterranean *O. taurus* (average abundance of 25.7 vs 0.29%, $p < 0.001$), and nearly so in EA *O. taurus* (average abundance of 6.8 vs 0.03%, $p = 0.117$) when compared to the corresponding native species of that region.

Wolbachia *in Dung Beetles*

Earlier studies have failed to identify *Wolbachia* as a member of the *O. taurus* microbiota (Estes et al., 2013; John (Jack) Werren, personal communications). We identified *Wolbachia* is indeed present, at times at high abundance, in a subset of populations and species (Fig. 4, Fig. S4). Specifically, four different OTUs were classified as *Wolbachia* in our primary, rarefied dataset, and 33 in the full, un-rarefied data set. Two of these (OTUs 1, and 27) were common enough to be included in our cutoff of at least 5% relative abundance in at least one sample, while the two others (OTUs 175 and 387) exhibited ≤ 10 reads in most samples.

Specifically, *Wolbachia* was most prevalent in Eastern Australian *O. taurus*, and Mediterranean *Euoniticellus fulvus*. In EA *O. taurus*, OTU 1 dominated and was found at an average relative abundance of 47% (ranging from 0.01-71%; 95% confidence interval of 26.7-67.3%). The MED *E. fulvus* samples also carried heavy *Wolbachia* infection loads, where OTU 1 also predominated, with an average relative abundance of 26% (range 5-45%, 95% confidence interval of 17.2-35.5%). In contrast, the corresponding sympatric EA and MED populations (EA *O. australis* and MED *O. taurus*) did not show high prevalence of *Wolbachia* infection. Aside from one EA *O. australis* individual in which OTU 27 accounted for 20% of the total relative abundance, no other *O. australis* or MED *O. taurus* sample exhibited a *Wolbachia* OTU with over 0.01% relative abundance. Lastly, only a single individual (EUS *O. hecate*) was found to be completely *Wolbachia* free aside from the blank.

DISCUSSION

Host-associated microbes influence host fitness and health by shaping diverse aspects of host biology (McFall-Ngai et al., 2013), and hosts are thus predicted to evolve microbial relationships that maximize their fitness in a given environment (Mazel et al., 2018). However, when hosts colonize novel environments microbial partnerships may shift, for instance because original modes of microbial acquisition become disrupted, the relevance of specific microbiota for host fitness is altered, or novel microbial partners become available. Yet relatively few studies have examined how microbiome composition changes in natural populations when hosts colonize novel geographic regions. In this study, we leveraged the *Onthophagus* dung beetle system to determine to what extent microbiome assemblies shift during host introduction events and the significance of ancestral associations and geography in the structuring of microbial communities of introduced species. Below we discuss our results and their most important implications.

Onthophagus taurus *microbiota are structured by both evolutionary history and local environmental forces*

We find that microbiota associated with native Mediterranean *Onthophagus taurus* cluster most closely to those of native Mediterranean *Euoniticellus fulvus*. That is, even though there is relatively little microbial community variation within these populations - seen both graphically (in Fig. 3), and statistically – they emerge as each other's nearest neighbor in our analyses. Likewise, microbiota associated with exotic Eastern US *O. taurus* cluster more closely to those of *O. hecate*, a species native to the same region, than to *O. taurus* microbiota from other regions (Fig. 3). These observations suggest that local environmental conditions contribute to structuring the microbial compositions of our focal host taxa.

At the same time, we also observe patterns consistent with an influence of ancestral host microbiome relationships on host beetles collected in non-native environments. For example, six of the eight microbiota samples derived from non-native EA *O. taurus* cluster with the clade containing microbiota associated with native MED (*O. taurus* and *E. fulvus*) beetles, yet are more distinct from the majority of syntopic EA *O. australis* (Fig. 3). This result is particularly interesting given the artificial introduction program employed to introduce exotic beetles into Australia (Edwards, 2007). This effort included the surface sterilization of eggs and their subsequent rearing in artificial brood balls, two measures intended to eliminate or at least disrupt microbial transfer from field collected to quarantined and field-released individuals. Yet these measures notwithstanding, the majority of EA *O. taurus* microbial communities continue to most resemble the communities seen in their native Mediterranean range. This suggests that the quarantine procedures put in place either failed, or alternatively that EA *O. taurus* were able to reassemble microbial partners similar to those also utilized in their native MED region. The ability of host species to reliably guide the assembly of specific microbial communities has recently been noted in a number of study systems (e.g. insects: Brucker & Bordenstein, 2013; Brooks et al., 2016; rodents: Brooks et al., 2016; Kohl et al., 2018; other mammals: Groussin et al., 2017), yet the mechanisms underlying this ability remain to be determined in most instances.

Onthophagus taurus' introduction to the Eastern US in contrast, did not involve any quarantine measures and instead is believed to have resulted from the accidental release of a single and small founding population. Remarkably, it was this accidental introduction that was followed by a rapid post-introduction range expansion far exceeding that observed following the deliberate releases of *O. taurus* in Eastern Australia (as well as Western Australia and California; Silva et al., 2016). Importantly, climatic conditions now inhabited by *O. taurus* in the Eastern US

are significantly different than those observed for its native Mediterranean distribution (Silva et al. 2016). This raises the possibility that adoption of a Eastern US range-specific microbiome could have contributed to the successful spread of *O. taurus* in this, but not other exotic ranges, similar to what has been suggested for other taxa (e.g. wasps: Hajek et al., 2013; Wooding et al., 2013; ants: Mueller et al., 2011; Cheng et al., 2019; and pine trees: Gundale et al., 2016). This explanation is consistent with our observation of a shift in the microbial communities of EUS *O. taurus* animals away from the ancestral MED population, and towards a closer resemblance to the EUS *O. hecate* population (Fig. 3), even though the sample sizes of our EUS populations limit the conclusions we can reach on this front. At the same time, we presently do not know how uniform the microbial communities associated with *O. taurus* throughout its native Mediterranean range are, and therefore can not exclude the possibility that founder effects could be contributing to the microbiome divergences observed between native and exotic *O. taurus* populations,

Microbial communities associated with native Eastern Australian *O. australis* showed a disjunct clustering, with two samples clustering with non-native Eastern Australian *O. taurus*, while the remaining five samples clustered with *O. hecate* native to the Eastern US. *Onthophagus australis* is unusual in that it is the only native species that can be found reliably and in appreciable numbers in cow dung, whereas the remaining > 200 native Australian *Onthophagus* species are largely restricted to marsupial dung (Monteith & Kenyon, 2011). It is interesting to speculate that the composition of the *O. australis* microbiome may be reflecting this resource shift toward microbiome members more typical of bona fide cow dung specialists, though future work on other native Australian *Onthophagus* is needed to assess this possibility.

Lastly, it is important to note that our understanding of the extent to which *Onthophagus* beetles rely on vertical transmission of their microbiota as compared to horizontal transmission from the environment remains incomplete. Previous work suggests that some fraction of the microbiome is indeed vertically inherited from mother to larvae, resulting in concordance between maternal microbial OTUs and those of larval offspring (Estes et al., 2013). Recent work also showed, however, that at least under benign laboratory conditions, several *Onthophagus* species are able to horizontally assemble functionally competent microbial communities even when their normally vertically transmitted microbiota is experimentally disrupted (Schwab et al., 2016; Parker et al., 2018). Yet the microbial community found in cow manure (the most common food source for *O. taurus*, and the other species used in this study) is rather distinct from that inhabiting the gut of *O. taurus* beetles feeding on that same manure (Estes et al., 2013). Evidence available to date thus suggests that *Onthophagus* beetles rely on a mix of both vertical transmission, and environmental filtering to construct their microbial communities, but more work is clearly needed to determine the relative contributions of horizontal and vertical transmission to microbiota assembly in this genus.

Putatively beneficial Dysgonomonas *symbionts are common among dung beetle species*

Even though each of the host populations we examined associated with several unique microbial taxa, some putatively beneficial symbionts were shared across all samples, such as the numerous OTUs classified as *Dysgonomonas*. *Dysgonomonas* bacteria were seen at overall similar abundances in every sample (Fig. 2, Fig. S3), and this genus also exhibited the greatest overall diversity in the dataset (12% of all classified OTUs). Insights into the biological significance of this genus outside the context of human health is limited, but common OTUs identified in this

study (8, 20, 32, 45, and 56) closely matched sequences previously identified as associated with guts of not only dung beetles (*O. taurus*: Estes et al., 2013; *Euoniticellus intermedius*: Shukla et al., 2016), but also fungus farming *Odontotermes* termites (Shinzato et al., 2005; Liu et al., 2013). Because *Dysgonomonas* is only found in *Odontotermes* fungal farms when termites are present it has been suggested that these bacteria play a role in controlling the spread of pathogenic fungus (Shinzato et al., 2005). Likewise, *Onthophagus* beetles must contend with attacks from entomopathogenic fungi such as *Metarhizium* ssp. throughout development and into adult life, and preliminary data support the hypothesis that maternally transmitted microbiota protect developing larvae from *Metarhizium* infections (Schwab et al. in prep.). Our results thus raise the possibility that *Dysgonomonas* may constitute a candidate bacterial genus for the possible synthesis of anti-fungal compounds able to protect their dung beetle hosts from fungal attacks. If correct, this might explain the maintenance of *Dysgonomonas* across diverse dung beetle species as well as native and recently established, exotic *O. taurus* populations. Future work must now focus on directly examining the precise functional significance of this microbial taxon, and address whether strong diversifying selection for anti-fungal compounds may be responsible for the great OTU diversity observed for this genus within and across *Onthophagus* species and populations.

Wolbachia *infections are common, but differentially abundant, among dung beetle populations and species*

Wolbachia are intracellular symbionts that are estimated to infect 20-66% of all insect species (Werren & Windsor, 2000; Hilgenboecker et al., 2008). These infections have diverse effects on host insects, ranging from beneficial (nutrient supplementation: Newton & Rice, 2019 and virus

protection: Hedges et al., 2008) to conditionally deleterious (Feminization, and killing of males: Werren et al., 2008). Despite the widespread distribution of *Wolbachia* infection among insects, only one study has so far detected *Wolbachia* in a dung beetle endemic to Thailand (*Onthophagus vaulogeri*: Sintupachee et al., 2006). Our results demonstrate that *Wolbachia* may be a common member of the dung beetle microbiota, though its abundance differs greatly between species and populations (Fig. 4, Fig. S4). Interestingly, the two populations in which *Wolbachia* infections are most prevalent belong to different species and derive from different geographic regions (EA *O. taurus* and MED *Euoniticellus fulvus*). In contrast, *Wolbachia* exhibited low abundances in both native MED and introduced EUS *O. taurus.* Population-specific differences in *Wolbachia* infection rates may, as already highlighted above, reflect founder effects: the MED *O. taurus* population examined in this study may not be reflective of the populations used to fuel EA and EUS introductions of this species. Alternatively, population-specific differences in *Wolbachia* infection rates may be a consequence of the divergent circumstances associated with both introductions. Recall that *O. taurus* introduced into Australia were surface sterilized as eggs, and then reared in artificial brood balls to avoid co-introducing exotic microbes (Edwards, 2007). As *Wolbachia* is an intracellular endosymbiont which aggregates in female ovaries and eggs, it likely escaped this sterilization procedure. Research in mosquitos has demonstrated that the native microbiome is able to contain the spread of *Wolbachia* infection, but that when the microbiome is disrupted by antibiotics, *Wolbachia* is more readily able to infect hosts and spread (Hughes et al., 2014). This raises the possibility that *Wolbachia* bacteria may be held at low levels by the native microbiome of MED *O. taurus,* but able to opportunistically proliferate in EA individuals. However, if correct, it remains unclear how EUS *O. taurus* were able to undergo a major divergence in their microbiome composition

yet retain low *Wolbachia* infection rates. Possibly, this difference in outcomes might be related to the sudden versus gradual microbiome disruption seen in EA and EUS introductions, respectively. Work is underway to address these and related questions, as well as to assess the potential phenotypic consequences of *Wolbachia* infections in Onthophagus beetles.

CONCLUSION

The data presented here offer a first glimpse into how the *Onthophagus taurus* microbiome is shaped by host-derived, and local environmental forces. We find that both factors structure the microbial communities of these animals, but that their relative importance is closely related to the unique introduction history of each population. Our results are thus compatible with both host-mediated maintenance of microbiomes across environments (i.e. phylosymbiosis, in EA *O. taurus*: Brooks et al., 2016), but also highlight the possibility of microbiome-mediated rapid local adaptation (in EUS *O. taurus*: Sudakaran et al., 2017). Even though more work is needed to further assess these implications, alongside the putative functional significance of key microbial taxa, our results underscore the promise of *Onthophagus* dung beetles as an exciting study system with which to explore the evolutionary ecology of symbiosis.

ACKNOWLEDGMENTS

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REFERENCES

Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., & Bordenstein, S. R. (2016). Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. *PLOS Biology*, *14*(11), e2000225.

Brucker, R. M., & Bordenstein, S. R. (2013). The Hologenomic Basis of Speciation : *Science*, *466*(August), 667–669.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME* J 6, 1621–1624.

Castrillo, L. A., Lee Jr, R. E., Lee, M. R., & Rutherford, S. T. (2000). Identification of ice-nucleating active *Pseudomonas fluorescens* strains for biological control of overwintering Colorado potato beetles (Coleoptera: Chrysomelidae). *Journal of economic entomology*, *93*(2), 226-233.

Cheng, D., Chen, S., Huang, Y., Pierce, N. E., Id, M. R., Yang, F., … Id, Y. X. (2019). Symbiotic microbiota may reflect host adaptation by resident to invasive ant species. *PLoS Pathogens*, 1–22.

Chu, C., Murdock, M. H., Jing, D., Won, T. H., Chung, H., Kressel, A. M., ... & Bessman, N. J. (2019). The microbiota regulate neuronal function and fear extinction learning. *Nature*, *574*(7779), 543-548.

De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D. H., … Carballa, M. (2016). Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. *Biotechnology for Biofuels*.

Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23(1), 38–47.

Edwards, P. (2007) Introduced Dung Beetles in Australia 1967-2007, 1–66.

Emelianoff, V., Chapuis, E., le Brun, N., Chiral, M., Moulia, C., and Ferdy, J. B. (2008) A Survival- Reproduction Trade-Off in Entomopathogenic Nematodes Mediated by Their Bacterial Symbionts. *Evolution*, 932-942

Estes, A. M., Hearn, D. J., Snell-Rood, E. C., Feindler, M., Feeser, K., Abebe, T., … Moczek, A. P. (2013). Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS ONE*, *8*(11), 1–15.

Floate, K. D., Watson, D. W., Weiss, R. M., & Olfert, O. (2017). Bioclimatic analyses for the distributions of *Onthophagus nuchicornis*, *Onthophagus taurus*, and *Digitonthophagus gazella* (Coleoptera: Scarabaeidae) in North America. *Canadian Entomologist*, *149*(4), 504–524.

Foray, V., Perez-Jimenez, M. M., Fattouh, N., & Landmann, F. (2018). Wolbachia control stem cell behavior and stimulate germline proliferation in filarial nematodes. *Developmental cell*, *45*(2), 198-211.

Frank, K., Brückner, A., Hilpert, A., Heethoff, M., & Blüthgen, N. (2017). Nutrient quality of vertebrate dung as a diet for dung beetles. *Scientific Reports*, *7*(1), 1–12.

Gilbert, S. F. (2019). Developmental symbiosis facilitates the multiple origins of herbivory. *Evolution & development*, e12291.

Goidanich, A., & Malan, C. E. (1962). *Sulla fonte di alimentazione e sulla microflora aerobica del nido pedotrofico e dell'apparato digerente delle larve di scarabei coprogagi:(Coleoptera scarabaeidae)*.

Groussin, M., Mazel, F., Sanders, J. G., Smillie, C. S., Lavergne, S., Thuiller, W., & Alm, E. J. (2017). Unraveling the processes shaping mammalian gut microbiomes over evolutionary time. *Nature Communications*, *8*.

Gundale, M. J., Almeida, J. P., Wallander, H., Wardle, D. A., Kardol, P., Nilsson, M. C., … Austin, A. (2016). Differences in endophyte communities of introduced trees depend on the phylogenetic relatedness of the receiving forest. *Journal of Ecology*, *104*(5), 1219–1232.

Hajek, A. E., Nielsen, C., Kepler, R. M., Long, S. J., & Castrillo, L. (2013). Fidelity Among *Sirex* Woodwasps and Their Fungal Symbionts. *Microbial Ecology*, *65*(3), 753–762.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science*, *322*(5902), 702-702.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with *Wolbachia*?–a statistical analysis of current data. *FEMS microbiology letters*, *281*(2), 215-220.

Holter, P. (2016). Herbivore dung as food for dung beetles: elementary coprology for entomologists. *Ecological Entomology*, *41*(4), 367–377.

Hooper, L. V., and Gordon, J. I. (2001) Commensal host-bacterial relationships in the gut. *Science*, 292(5519), 1115-1118.

Huang, S., Sheng, P., & Zhang, H. (2012). Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *International journal of molecular sciences*, *13*(3), 2563-2577.

Hughes, G. L., Dodson, B. L., Johnson, R. M., Murdock, C. C., Tsujimoto, H., Suzuki, Y., ... & Sakamoto, J. M. (2014). Native microbiome impedes vertical transmission of *Wolbachia*

in *Anopheles* mosquitoes. *Proceedings of the National Academy of Sciences*, *111*(34), 12498-12503.

Killham, K., & Prosser, J. I. (2007). *THE PROKARYOTES Volume 6: Proteobacteria: Gamma Subclass*.

Kohl, K. D., Dearing, M. D., & Bordenstein, S. R. (2018). Microbial communities exhibit host species distinguishability and phylosymbiosis along the length of the gastrointestinal tract. *Molecular Ecology*, *27*(8), 1874–1883.

Leonardo, T. E., and Mondor, E. B. (2006) Symbiont modifies host life-history traits that affect gene flow. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1590), 1079- 1084.

Liu, N., Zhang, L., Zhou, H., Zhang, M., Yan, X., Wang, Q., ... & Zhou, Z. (2013). Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (*Odontotermes yunnanensis*). *PLoS One*, *8*(7), e69184.

Mazel, F., Davis, K. M., Loudon, A., Kwong, W. K., Groussin, M., & Parfrey, L. W. (2018). Is Host Filtering the Main Driver of Phylosymbiosis across the Tree of Life? *MSystems*, *3*(5), e00097-18.

McFall-Ngai, M. J. (2014). The importance of microbes in animal development: lessons from the squid-*vibrio* symbiosis. *Annual Review of Microbiology*, *68*, 177-194.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V, Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229–3236.

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, 8(4), e61217.

Morimoto, J., Simpson, S. J., & Ponton, F. (2017). Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, *13*, 20160966.

Monteith, G., & Kenyon, T. (2011). Report on a survey of dung beetles (Coleoptera: Scarabaeinae) from the Moggill Creek Catchment Brisbane. Brisbane.

Mueller, U. G., Mikheyev, A. S., Hong, E., Sen, R., Warren, D. L., Solomon, S. E., … Juenger, T. E. (2011). Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant-fungus symbiosis. *Proceedings of the National Academy of Sciences*, *108*(10), 4053–4056.

Newton, I. L., & Rice, D. W. (2019). The Jekyll and Hyde symbiont: could Wolbachia be a nutritional mutualist?. *Journal of bacteriology*.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., ... & Stevens, M. H. H. (2018). vegan: Community Ecology Package. R package version 2.5-2. 2018.

Parker, E. S., Dury, G. J., & Moczek, A. P. (2018). Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles. *Ecological Entomology*.

Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 -- Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE, 5(3):e9490.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(D1), D590-D596.

R Core Team (2013). R: A language and environment for statistical computing.

Rosenberg, E., & Zilber-Rosenberg, I. (2013). The hologenome concept: Human, animal and plant microbiota. In *The Hologenome Concept: Human, Animal and Plant Microbiota*.

Rougon, D., Rougon, C., Levieux, J., & Trichet, J. (1990). Variations in the amino-acid content in zebu dung in the Sahel during nesting by dung-beetles (Coleoptera, Scarabaeidae). *Soil Biology and Biochemistry*, *22*(2), 217–223.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., ... & Sahl, J. W. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, *75*(23), 7537-7541.

Schwab, D. B., Riggs, H. E., Newton, I. L. G., & Moczek, A. P. (2016). Developmental and Ecological Benefits of the Maternally Transmitted Microbiota in a Dung Beetle. *The American Naturalist*, *188*(6), 000–000.

Shikuma, N. J., Pilhofer, M., Weiss, G. L., Hadfield, M. G., Jensen, G. J., & Newman, D. K. (2014). Marine tubeworm metamorphosis induced by arrays of bacterial phage tail–like structures. *Science*, *343*(6170), 529-533.

Shinzato, N., Muramatsu, M., Watanabe, Y., & Matsui, T. (2005). Termite-regulated fungal monoculture in fungus combs of a macrotermitine termite *Odontotermes formosanus*. *Zoological science*, 22(8), 917-923.

Shukla, S. P., Sanders, J. G., Byrne, M. J., & Pierce, N. E. (2016). Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular Ecology*, *25*(24), 6092–6106.

Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B., & Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS microbiology reviews*, *35*(4), 652-680.

Silva, D. P., Vilela, B., Buzatto, B. A., Moczek, A. P., & Hortal, J. (2016). Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, 18(11), 3137–3148.

Sintupachee, S., Milne, J. R., Poonchaisri, S., Baimai, V., & Kittayapong, P. (2006). Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. *Microbial Ecology*, *51*(3), 294–301.

Sneed, J. M., Sharp, K. H., Ritchie, K. B., & Paul, V. J. (2014). The chemical cue tetrabromopyrrole from a biofilm bacterium induces settlement of multiple Caribbean corals. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1786), 20133086.

Sommer, F., & Bäckhed, F. (2013). The gut microbiota—masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227.

Sudakaran, S., Kost, C., & Kaltenpoth, M. (2017). Symbiont Acquisition and Replacement as a Source of Ecological Innovation. *Trends in Microbiology*.

Tarasov, S. I., and Kabakov, O. N. (2010) Two new species of *Onthophagus* (Coleoptera: Scarabaeidae) from Indochina, with a discussion of some problems with the classification of Serrophorus and similar subgenera. *Zootaxa*, 2344, 17-28.

Vulinec, K., & Eudy, S. P. (1993). A southern range extension for the introduced dung beetle *Onthophagus taurus* Schreber (Coleoptera: Scarabaeidae). *Coleopterists Bulletin*, *47(2)*(January 1993), 129–130.

Vuong, H. E., Yano, J. M., Fung, T. C., & Hsiao, E. Y. (2017). The Microbiome and Host Behavior. *Annual Review of Neuroscience*, *40*(1), 21–49

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

Werren, J. H., & Windsor, D. M. (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1450), 1277-1285.

Whalan, S., & Webster, N. S. (2014). Sponge larval settlement cues: the role of microbial biofilms in a warming ocean. *Scientific Reports*, *4*, 4072.

White, J. R., Nagarajan, N., & Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS computational biology*, 5(4), e1000352.

Wickham, H. (2016). ggplot2: elegant graphics for data analysis. Springer.

Wooding, A. L., Wingfield, M. J., Hurley, B. P., Garnas, J. R., De Groot, P., & Slippers, B. (2013). Lack of fidelity revealed in an insect-fungal mutualism after invasion. *Biology Letters*, *9*(4).

FIGURES AND LEGENDS

Figure 1:

Native and introduced ranges of *Onthophagus taurus* used in this study. Animals pictured are *O. taurus* (on the left), and corresponding native, syntopic, beetles selected from each region (on the right). Native species paired with *O. taurus* at each location are *O. hecate* in the Eastern US, *Euoniticellus fulvus* in the Mediterranean, and *O. australis* in Eastern Australia.

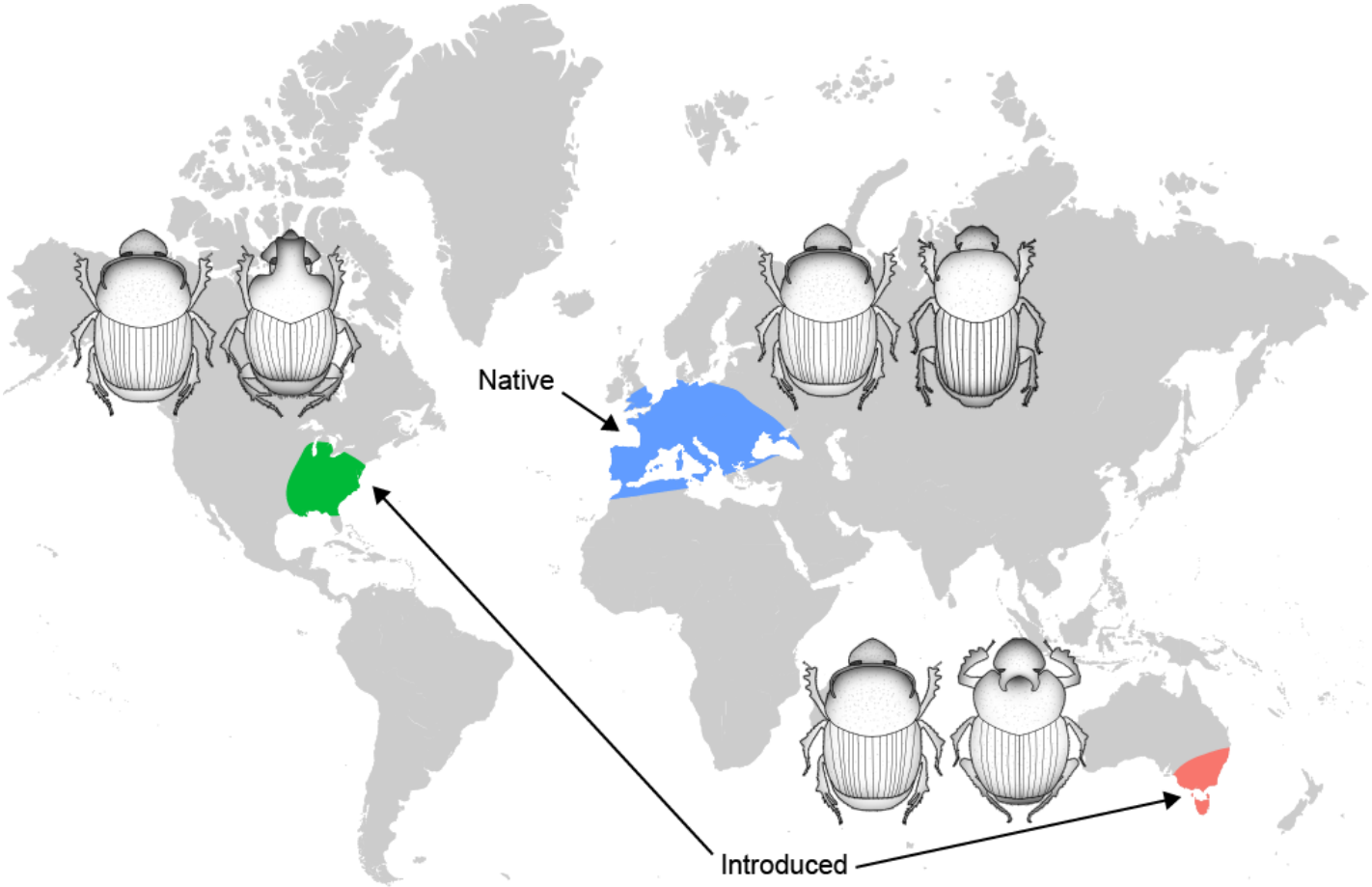


Figure 2:

Bar plot of relative average abundances of microbial taxa for each sample group. Colored blocks represent bacterial OTUs found at ≥ 5% relative abundance in at least one sample. All other rare OTUs not making this cutoff are binned into the gray bars for < 5% relative abundance. The key is sorted by phylum and then class (bolded), followed by each OTU number and the genus of that OTU.

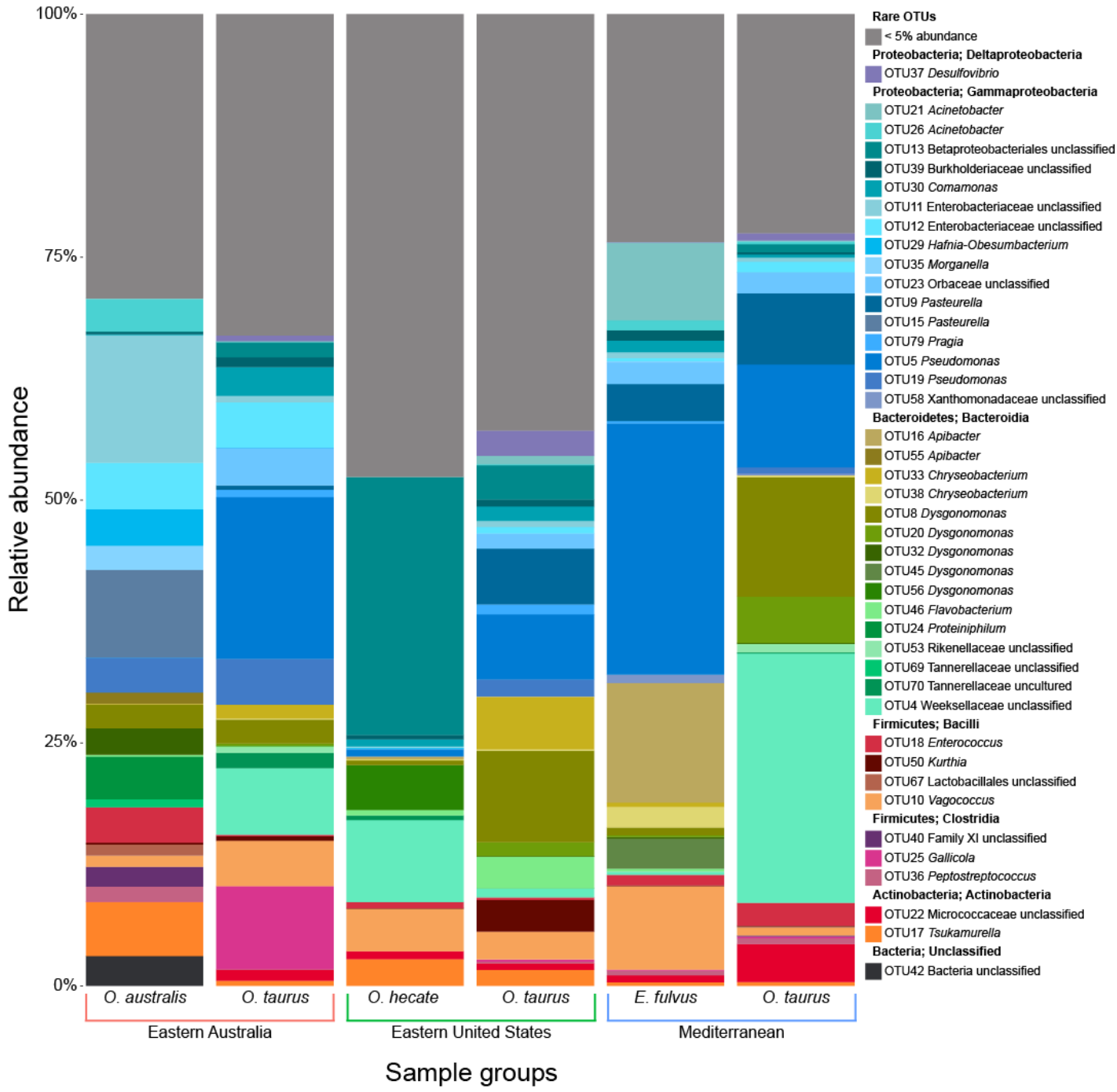

100%
75%
50%
25%
0%
Relative abundance
O. australis
O. taurus
O. hecate
O. taurus
E. fulvus
O. taurus
Eastern Australia
Eastern United States
Mediterranean
Sample groups
Rare OTUs
< 5% abundance
Proteobacteria; Deltaproteobacteria
OTU37 Desulfovibrio
Proteobacteria; Gammaproteobacteria
OTU21 Acinetobacter
OTU26 Acinetobacter
OTU13 Betaproteobacteriales unclassified
OTU39 Burkholderiaceae unclassified
OTU30 Comamonas
OTU11 Enterobacteriaceae unclassified
OTU12 Enterobacteriaceae unclassified
OTU29 Hafnia-Obesumbacterium
OTU35 Morganella
OTU23 Orbaceae unclassified
OTU9 Pasteurella
OTU15 Pasteurella
OTU79 Pragia
OTU5 Pseudomonas
OTU19 Pseudomonas
OTU58 Xanthomonadaceae unclassified
Bacteroidetes; Bacteroidia
OTU16 Apibacter
OTU55 Apibacter
OTU33 Chryseobacterium
OTU38 Chryseobacterium
OTU8 Dysgonomonas
OTU20 Dysgonomonas
OTU32 Dysgonomonas
OTU45 Dysgonomonas
OTU56 Dysgonomonas
OTU46 Flavobacterium
OTU24 Proteiniphilum
OTU53 Rikenellaceae unclassified
OTU69 Tannerellaceae unclassified
OTU70 Tannerellaceae uncultured
OTU4 Weeksellaceae unclassified
Firmicutes; Bacilli
OTU18 Enterococcus
OTU50 Kurthia
OTU67 Lactobacillales unclassified
OTU10 Vagococcus
Firmicutes; Clostridia
OTU40 Family XI unclassified
OTU25 Gallicola
OTU36 Peptostreptococcus
Actinobacteria; Actinobacteria
OTU22 Micrococcaceae unclassified
OTU17 Tsukamurella
Bacteria; Unclassified
OTU42 Bacteria unclassified

Figure 3:

A) Non-metric MultiDimensional Scaling (NMDS) plot of unweighted UniFrac distances for each sample. Points are colored by region, with different shapes for each species. Colored ellipses with confidence levels of 90% were generated for the EA and MED samples, but not for EUS as there were too few datapoints. B) Cluster diagram of all samples based on unweighted UniFrac and the Ward.D2 clustering algorithm. Sample names colored by sample region.

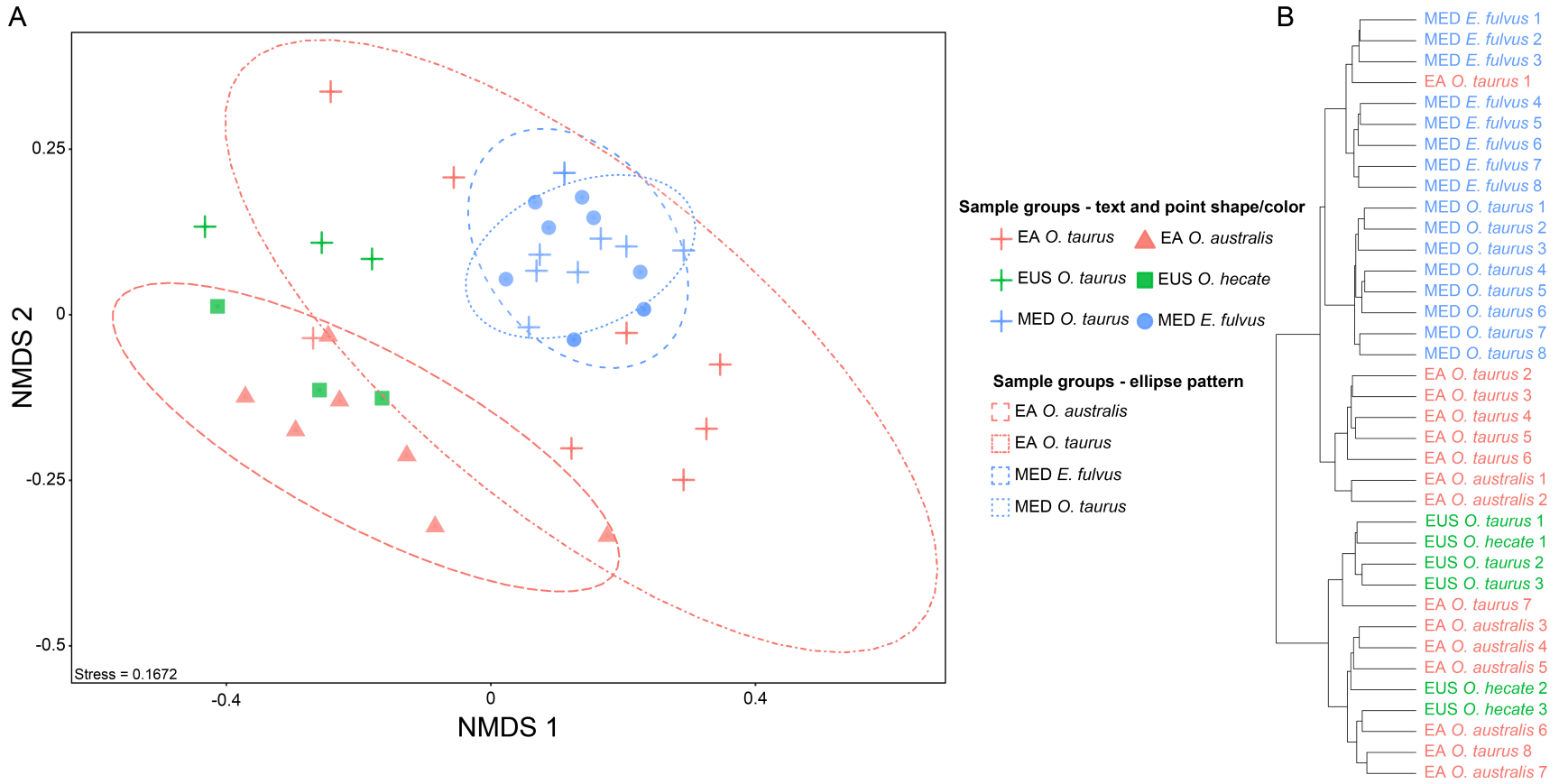
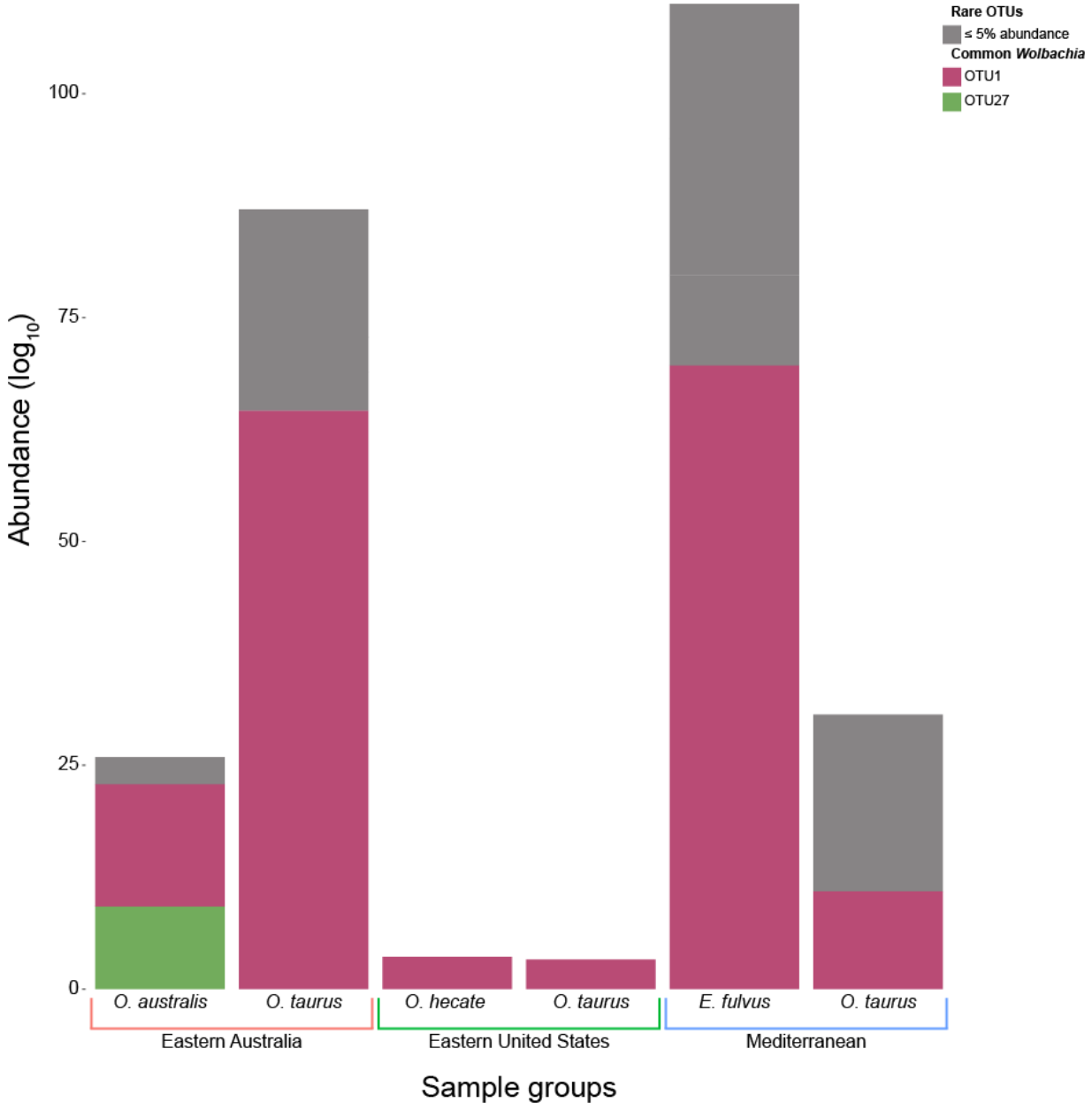


Figure 4:

Log_{10} transformed bar plot showing the relative abundance of common *Wolbachia* OTUs found in each sample group. Commonness criteria chosen was ≥ 5% relative abundance in at least one sample – all other OTUs falling below this cutoff were binned into the gray bars. Colored brackets below species names illustrate region of origin for the animals. Log_{10} transformation was used to increase legibility by correcting for large differences in abundance between samples.



SUPPLEMENTARY FIGURES AND LEGENDS

Figure S1:

Rarefaction curves for each sample in the primary dataset, from zero reads until the rarefication cutoff of 22,646 reads. The slopes of the majority of samples begin leveling off around this cutoff – though some continue to increase rapidly past this point. This indicates that our chosen rarefication cutoff achieved adequate sampling depth, despite the apparent high complexity of the dung beetle microbiome.

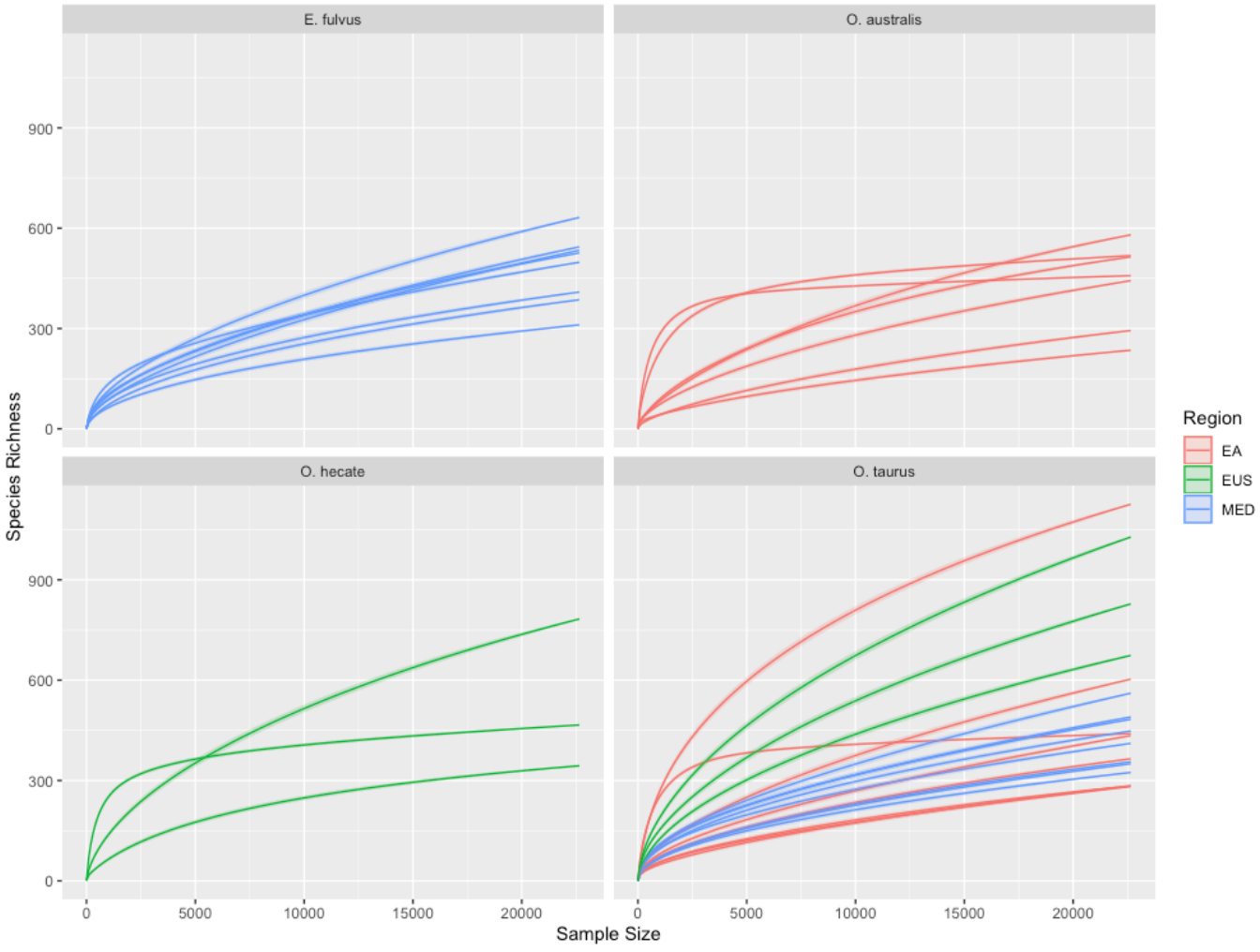


Figure S2:

Estimated Chao1, Shannon, and Simpson alpha diversity indices for all samples in the primary dataset. Colored brackets below species names indicate region of origin for each sample group. Letters above each boxplot indicate results of ANOVA tests for significant differences in diversity estimates between sample groups. Only the Chao1 estimate for EUS *Onthophagus taurus* samples was significantly different from other groups ($p < 0.001$).

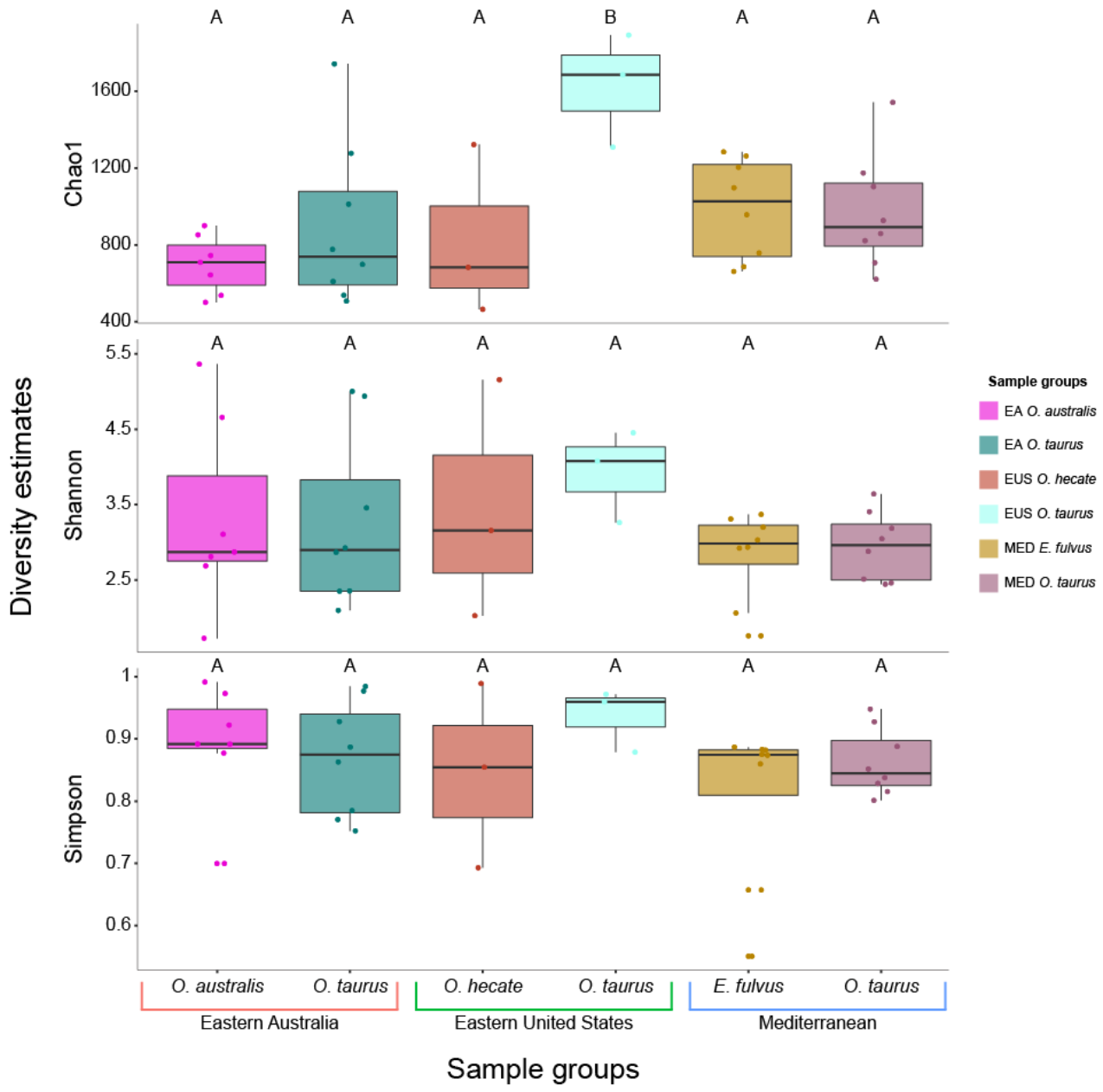


Figure S3:

Bar plot of relative average abundances of microbial taxa for each sample group, with microbial taxa grouped by genus. Colored blocks represent bacterial OTUs found at ≥ 5% relative abundance in at least one sample. All other rare OTUs not making this cutoff are binned into the gray bars for < 5% relative abundance. The key is sorted by phylum and then class (bolded), followed by each genus.

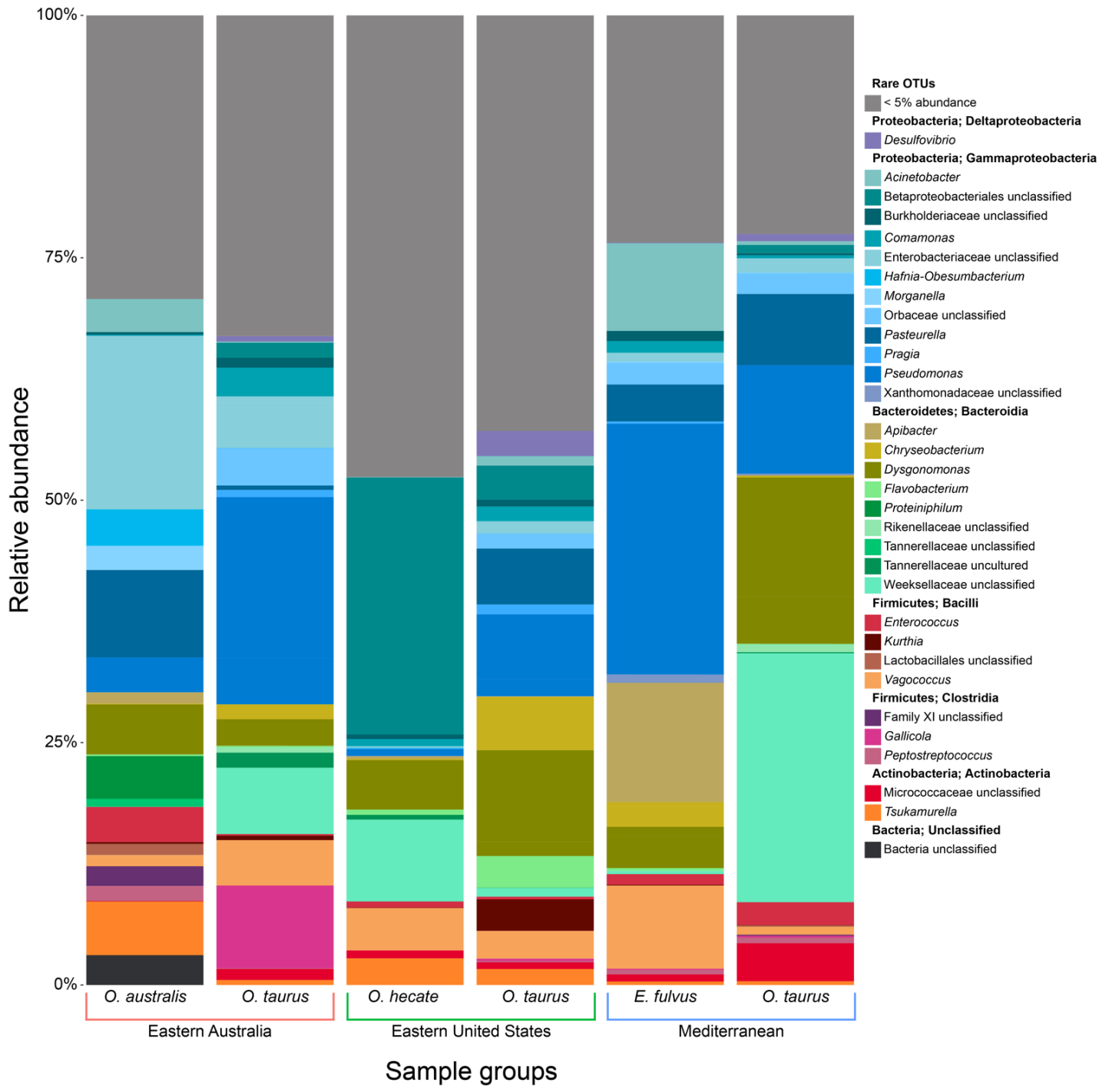
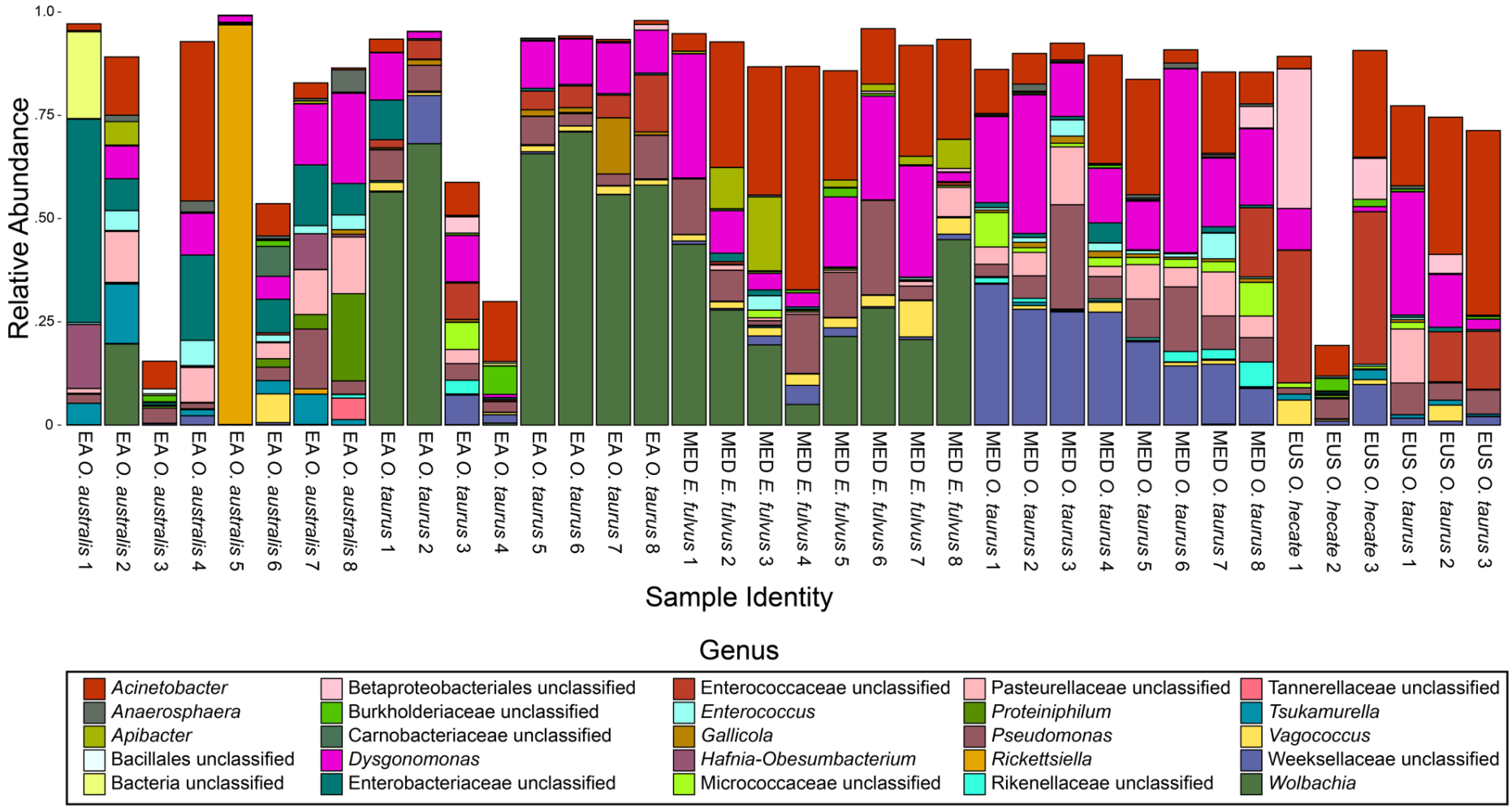

100%
75%
50%
25%
0%
Relative abundance
O. australis
O. taurus
O. hecate
O. taurus
E. fulvus
O. taurus
Eastern Australia
Eastern United States
Mediterranean
Sample groups
Rare OTUs
< 5% abundance
Proteobacteria; Deltaproteobacteria
Desulfovibrio
Proteobacteria; Gammaproteobacteria
Acinetobacter
Betaproteobacteriales unclassified
Burkholderiaceae unclassified
Comamonas
Enterobacteriaceae unclassified
Hafnia-Obesumbacterium
Morganella
Orbaceae unclassified
Pasteurella
Pragia
Pseudomonas
Xanthomonadaceae unclassified
Bacteroidetes; Bacteroidia
Apibacter
Chryseobacterium
Dysgonomonas
Flavobacterium
Proteiniphilum
Rikenellaceae unclassified
Tannerellaceae unclassified
Tannerellaceae uncultured
Weeksellaceae unclassified
Firmicutes; Bacilli
Enterococcus
Kurthia
Lactobacillales unclassified
Vagococcus
Firmicutes; Clostridia
Family XI unclassified
Gallicola
Peptostreptococcus
Actinobacteria; Actinobacteria
Micrococcaceae unclassified
Tsukamurella
Bacteria; Unclassified
Bacteria unclassified

Figure S4:

Bar plots of relative abundances of microbial taxa for each sample individually, with bars colored by microbial genus. X-axis displays each sample name, and the Y- axis displays the relative abundance of each common microbial genus (with separate OTUs grouped by genus) found at ≥ 5% relative abundance in at least one sample. This dataset includes OTUs assigned as *Wolbachia* (shown in dark green).


Relative Abundance
1.0
.75
.50
.25
0
EA O. australis 1
EA O. australis 2
EA O. australis 3
EA O. australis 4
EA O. australis 5
EA O. australis 6
EA O. australis 7
EA O. australis 8
EA O. taurus 1
EA O. taurus 2
EA O. taurus 3
EA O. taurus 4
EA O. taurus 5
EA O. taurus 6
EA O. taurus 7
EA O. taurus 8
MED E. fulvus 1
MED E. fulvus 2
MED E. fulvus 3
MED E. fulvus 4
MED E. fulvus 5
MED E. fulvus 6
MED E. fulvus 7
MED E. fulvus 8
MED O. taurus 1
MED O. taurus 2
MED O. taurus 3
MED O. taurus 4
MED O. taurus 5
MED O. taurus 6
MED O. taurus 7
MED O. taurus 8
EUS O. hecate 1
EUS O. hecate 2
EUS O. hecate 3
EUS O. taurus 1
EUS O. taurus 2
EUS O. taurus 3
Sample Identity
Genus
Acinetobacter
Anaerosphaera
Apibacter
Bacillales unclassified
Bacteria unclassified
Betaproteobacteriales unclassified
Burkholderiaceae unclassified
Carnobacteriaceae unclassified
Dysgonomonas
Enterobacteriaceae unclassified
Enterococcaceae unclassified
Enterococcus
Gallicola
Hafnia-Obesumbacterium
Micrococcaceae unclassified
Pasteurellaceae unclassified
Proteiniphilum
Pseudomonas
Rickettsiella
Rikenellaceae unclassified
Tannerellaceae unclassified
Tsukamurella
Vagococcus
Weeksellaceae unclassified
Wolbachia

CHAPTER 6

Discussion

OVERVIEW

A large body of work demonstrates the diverse benefits that hosts derive from their microbial partners (as reviewed in McFall-Ngai et al., 2013). These findings have motivated the complementary hypotheses that host diversification and adaptive radiations may in part be shaped by the symbiotic partnerships hosts form, and that evolutionary processes may also act on teams composed of hosts and their microbiota to ultimately drive these diversification events (Sudakaran et al., 2017). While evidence supports these hypotheses in at least some circumstances and systems, the general applicability of this framework has so far been largely untested and remains primarily theoretical. In order to satisfyingly investigate the role of the microbiome in host diversification it is clear that new hands-on experimental approaches and model systems need to be developed.

In this dissertation, I aimed to address this opening by developing *Onthophagus* dung beetles as a new, powerful system in which to investigate the role of the microbiome in host adaptation and evolution. Through my work, I have employed a manipulative approach which enabled me to exchange the microbiome between *Onthophagus* individuals. In turn, this allowed me to show that distantly related species develop non-interchangeable microbial communities which persist across generations (chapter 2). Using this same experimental approach, I then found that host-microbiome relationships can diverge even between ecologically similar, sympatric, sister species (chapter 3). I then applied these techniques, with an added temperature manipulation, to more directly investigate the role of the microbiome in the adaptive radiation of one recently invasive, and still rapidly expanding *Onthophagus* species. Here I found that the

microbiome might constrain, rather than facilitate such rapid expansion (chapter 4). Finally, I used bacterial 16S sequencing to compare and contrast the microbiomes of native and introduced *Onthophagus* species, finding that these microbial communities are structured by both local environmental forces and maintained ancestral associations (chapter 5). Together, the chapters of my dissertation were designed to both expand the use of the *Onthophagus* system for studies into the mechanisms and consequences of host-microbiome interactions, and to expand our fundamental understanding of the evolutionary ecology of symbiosis.

THE EVOLUTION OF *ONTHOPHAGUS* SYMBIOSIS

The crucial role microbial symbionts play in regulating many aspects of host health, development, behavior, and homeostasis is well appreciated across much of the tree of life (McFall-Ngai et al., 2013). One area where this appreciation has been slower to develop, however, is the field of evolutionary biology. Many evolutionary biologists regard as anathema the idea that selection could act upon anything other than individual organisms, and subsequently disregard the notion of hosts and their symbionts forming teams to better tackle selective challenges – though support for this idea may be building within the broader evolutionary community (Gilbert et al., 2015; Sudakaran et al., 2017; Hu et al., 2019). Regardless, the current dearth of experimental evidence available to put this notion to the test necessitates the further development of experimental study systems and execution of manipulative studies.

Chapter 2 addressed these and related questions by investigating the transgenerational consequences of microbial symbiont disruption. By exchanging the gut microbiome of two beetle species, *Onthophagus gazella* and *O. sagittarius*, I was able to show that 1) these species

have evolved relationships with their microbiome which are not substitutable through inoculation with another species microbiome, and 2) that disruption of these relationships leads to negative fitness consequences able to persist across generations. Thus, this study was among the first to demonstrate the potential for the extra-genetic inheritance of microbiome members to directly impact host evolution (Sharon et al., 2010; Morimoto et al., 2017). This finding then provided important support for a more expansive view of the microbial environment as an agent able to directly impact the fitness, and evolutionary trajectories of host organisms.

While generating intriguing findings, the work presented in chapter 2 had the limitation of focusing on two geographically distinct, and evolutionarily distant species (Emlen *et al.*, 2005; Breeschoten *et al.*, 2016). This left unanswered the question whether the observed lack of exchangeability of microbiota across the two host species was simply a side effect of ecological differentiation and/or distant evolutionary relatedness. I used chapter 3 to address this issue by performing pedestal microbiome exchanges between the sister-species *Onthophagus vacca* and *O. medius*, thus illustrating that microbiome mediated divergence in fitness-relevant host traits can be found even between two otherwise similar species which share largely overlapping central-European distributions, are often collected from the same dung pads, and readily form low-fitness hybrids in captivity (Roessner et al., 2010; Roy et al., 2016). Additionally, as in chapter 2 the effects of pedestal swapping were found to be asymmetric between species, further underscoring the complexity and species-specific nature of host-microbiome relationships in *Onthophagus*.

Taken together, these two chapters illustrate that host-microbiome interactions can diverge across a variety of evolutionary and ecological scenarios, and that following divergence these newly distinct microbial communities are able to directly affect the evolution of their hosts.

Through these conclusions, the results of these chapters provide important empirical data supporting the, so far, largely theoretical hologenome framework. This theory – essentially an expansion and formalization of the general notion that hosts and their microbes can form "teams", as outlined earlier – posits that hosts and their associated, inherited microbes can be conceptualized as a single biological unit upon which selection can act to drive evolutionary outcomes (Bordenstein & Theis, 2015). Key within this concept is the idea that the extra-genetic inheritance of microbiota can and does impact the evolutionary trajectories of hosts. And that, much like the accumulation of mutations in the nuclear genome, changes to the inherited microbiome have the ability to alter the fitness, and ultimate evolutionary outcomes of their hosts. While the insights from these chapter provided intriguing support for this framework, they are by no means conclusive support and much more work remains to be done. Specifically, in *Onthophagus* it remains unclear the extent to which the changes to host-microbiome interactions uncovered thus far were a driver of host adaptation, rather than a consequence thereof. To address this question, I focused the last two chapters of my dissertation on exploring the role of host-microbe interactions in structuring the rapid, local adaptation of newly invasive species.

THE ECOLOGY OF *ONTHOPHAGUS* SYMBIOSIS

To begin exploring the ecological dimension of *Onthophagus* symbiosis, I employed a DNA sequencing approach in chapter 5 to analyze the whole-body microbial communities of *Onthophagus taurus* beetles collected from their native Mediterranean, and two exotic ranges. By comparing these sequence data to those collected from sympatric dung beetle species in these same regions I was able to show that the *O. taurus* microbiome is structured by both local

microbe availability, and historical, ancestral relationships. Interestingly, this was found to be true even for *O. taurus* collected from Eastern Australia – animals which underwent strict quarantine, pedestal removal, and microbial sterilization procedures prior to their release into the wild (Edwards, 2007). Assuming these disruptions were as effective as reported, this finding thus highlighted the strength of the coadaptation between *Onthophagus* beetles and their microbiome. That is, even after the maternal transmission of microbes was disrupted in these animals, their descendants were able to reassemble functionally competent microbial communities from what was available in their new environment. Furthermore, the microbial communities these exotic *O. taurus* populations assembled were at least partly taxonomically distinct from those seen in both the native Mediterranean *O. taurus* populations from which these introduced animals were derived, and the corresponding native Australian population to which they were compared. And while this isn't the first study to show that invasive species assemble microbiome communities distinct from both their native and introduced locations (Gundale et al., 2016), it did serve to further highlight the large roles played by both evolutionary and ecological forces in structing the microbial communities of these animals. Uncovering this dual influence of both ancestral associations, and novel environmental pressures as structuring forces in *Onthophagus* microbiome assembly then raised the question of which of these forces – if either – is more important in structuring the local, rapid adaptation characteristic of introduced species.

To investigate this question, I performed pedestal swaps between two populations of *O. taurus* collected from the northern and southern extremes of their exotic Eastern US range (Michigan and Florida, respectively). Of the many places *O. taurus* has been introduced around the world, the Eastern US is both the only accidental introduction event, and the one which has resulted in the by far most extreme expansion of realized niche space (Silva et al., 2016). In just

50 years from their introduction into northern Florida, *O. taurus* has spread as far north as northern Michigan, a location significantly colder than their native range. This confluence of factors lead me to hypothesize that this rapid northern range expansion was mediated at least in part by the maintenance of a unique microbiome during this introduction event. And, though limited, research in other systems appears to support a role for the microbiome in mediating cold tolerance in insects (Mueller et al., 2011; Corbin et al., 2017; Renoz et al., 2019). However, contrary to my predictions, I found that pedestal microbiome swapping *did not* confer benefits consistent with local, rapid cold-temperature adaptation. Instead, I found that animals from both populations reared with the "wrong" pedestal outperformed animals reared with their own pedestal under cold conditions. This pattern of increased performance when exposed to a similar (in this case derived from the metapopulation), yet still distinct microbiome was consistent with what would be expected under enemy release dynamics. While historically employed to explain increased plant performance in non-native soils, the general idea that release from negative interactions leads to increased host performance also fits with host-microbiome interaction dynamics. It thus appears plausible in this case that the rapid post-introduction spread of *O. taurus* throughout the Eastern US was made possible not by the rapid adaptation of *beneficial*, co-adapted microbes, but by the replacement of deleterious microbes with novel associates.

Together, the four chapters of this dissertation suggest that when introduced to novel environments *Onthophagus* beetles are able to rapidly adapt by relying on both their historical, co-adapted microbial associates, and their ability to form flexible associations with novel, environmental microbes. If correct, this interpretation complements established theory: the notion that host organisms may rely on the key functions contributed by a "core" set of regularly inherited microbes, while maintaining the ability to rapidly adapt to new environments through

flexible associations with pools of environmental microbes, a position advanced in the context of the hologenome theory of evolution (Shapira et al., 2016; Lemanceau et al., 2017; Rosenberg & Zilber-Rosenberg, 2018). And while far from conclusive, the sum total of work presented in this dissertation represents, to the best of my knowledge, one of the few empirical demonstrations of support for diverse aspects of this particular framework. It remains to be seen, however, how robust these results are across diverse species and contexts and how well they represent a general theme in biological systems.

THE FUTURE OF EVOLUTIONARY ECOLOGY OF SYMBIOISIS RESEARCH IN *ONTHOPHAGUS*

When I began my dissertation research, we knew that *Onthophagus* beetles vertically transmitted gut microbes through the pedestal (Estes et al., 2013), that gut microbes were developmentally important, in particular under stressful conditions, and that host species had diverged in their reliance on said microbiota (Schwab et al., 2016). As I write this, we now also know that: 1) *Onthophagus* form species-specific relationships with their gut microbiotas, disruptions to which can have negative, transgenerational fitness effects (Parker et al., 2019); 2) these species-specific relationships can evolve even between sister species possessing nearly identical autecologies (Parker et al., *in review*); that both ecological and evolutionary forces play a strong role in structuring *Onthophagus* microbiome assembly following introduction events (Parker et al., 2020); and that the invasion success of *Onthophagus taurus* beetles may be enhanced by post-introduction release from negative microbiome pressures (Parker & Moczek, 2020). These

advances have both greatly expanded our knowledge of this study system, and also opened up new avenues of research into the evolutionary ecology of *Onthophagus* symbioses.

For example, chapters 2 and 3 highlight that host-microbiome coadaptation can been seen at two extremes of evolutionary distance in *Onthophagus* – but it remains to been seen if the fitness detriments incurred by pedestal swapping increase linearly with the evolutionary time between species pairs, as predicted by the phylosymbiosis framework (Brooks et al., 2016; Lim & Bordenstein, 2020). With so many experimentally available species at varying levels of evolutionary relatedness, coupled with now well-developed microbiome swapping protocols, *Onthophagus* seems an ideal system for future researchers to methodically and rigorously put the predictions of phylosymbiosis to the test. Similarly, the large number of successful *Onthophagus* introduction events around the globe opens the door to a more thorough and systematic exploration of both post-introduction microbiome assembly dynamics, and the role of the microbiome in enabling introduction success. Chapters 4 and 5 gave us hints as to what the answers to these questions might be in this genus, but truly satisfying answers can only be achieved by building upon this early work.

Finally, chapter 5. represents the first modern, high-quality microbiome sequencing effort across several *Onthophagus* species –. As part of this effort, this project identified for the first time in *Onthophagus* a number of particularly interesting microbial taxa, such as the reproductive parasite *Wolbachia* and the putative anti-fungal compound producing bacteria *Dysgonomonas*. Identification of taxa such as these has opened the door to future work in this system focused not just on the function of microbiome as a whole, but rather the functions of its individual members. Ultimately, it is my hope that the work presented in this dissertation will facilitate the development and application of experimental approaches to further advance our

understanding of the nature and consequences of microbial symbiosis in the *Onthophagus* system.

REFERENCES

Bordenstein, S. R., & Theis, K. R. (2015). Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. *PLoS Biology*, *13*(8), 1–23.

Breeschoten, T., Doorenweerd, C., Tarasov, S., and Vogler, A. P. (2016) Phylogenetics and biogeography of the dung beetle genus Onthophagus inferred from mitochondrial genomes. *Molecular phylogenetics and evolution*, 105, 86-95.

Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., & Bordenstein, S. R. (2016). Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. *PLOS Biology*, *14*(11), e2000225.

Corbin, C., Heyworth, E. R., Ferrari, J., & Hurst, G. D. D. (2017). Heritable symbionts in a world of varying temperature. *Heredity*, *118*(1), 10–20.

Edwards, P. (2007) Introduced Dung Beetles in Australia 1967-2007, 1–66.

Emlen, D. J., Marangelo, J., Ball, B., and Cunningham, C. W. (2005) Diversity in the Weapons of Sexual Selection: Horn Evolution in the Beetle Genus *Onthophagus* (coleoptera: Scarabaeidae). *Evolution*, 59(5), 1060–1084.

Gilbert, S. F., Bosch, T. C. G., & Ledón-Rettig, C. (2015). Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. *Nature Reviews Genetics*, *16*(10), 611–622.

Gundale, M. J., Almeida, J. P., Wallander, H., Wardle, D. A., Kardol, P., Nilsson, M. C., … Austin, A. (2016). Differences in endophyte communities of introduced trees depend on the phylogenetic relatedness of the receiving forest. *Journal of Ecology*, 104(5), 1219–1232.

Hu, Y., Linz, D. M., Parker, E. S., Schwab, D. B., Casasa, S., Macagno, A. L. M., & Moczek, A. P. (2019). Developmental bias in horned dung beetles and its contributions to innovation, adaptation, and resilience. *Evolution & Development*, 1–16.

Hurst, G. D. D. (2017). Extended genomes: symbiosis and evolution. *Interface Focus*, *7*(5), 20170001.

Lemanceau, P., Blouin, M., Muller, D., & Moënne-Loccoz, Y. (2017). Let the Core Microbiota Be Functional. *Trends in Plant Science*, *xx*.

Lim, S. J., & Bordenstein, S. R. (2020). An introduction to phylosymbiosis. *Proceedings of the Royal Society B: Biological Sciences*, 287.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V, Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229–3236.

Morimoto, J., Simpson, S. J., & Ponton, F. (2017) Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, 13, 20160966.

Mueller, U. G., Mikheyev, A. S., Hong, E., Sen, R., Warren, D. L., Solomon, S. E., … Juenger, T. E. (2011). Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant-fungus symbiosis. *Proceedings of the National Academy of Sciences*, *108*(10), 4053–4056.

Parker, E. S., Dury, G. J., & Moczek, A. P. (2019). Transgenerational developmental effects of species-specific, maternally transmitted microbiota in Onthophagus dung beetles. *Ecological Entomology*, *44*(2), 274-282.

Parker, E. S., & Moczek, A. P. (2020). Don’t stand so close to me: Microbiota-facilitated enemy release dynamics in introduced Onthophagus taurus dung beetles. *Ecology and Evolution*.

Parker, E. S., Moczek, A. P., & Macagno, A. L. M. Reciprocal microbiome transplants differentially rescue fitness in two syntopic dung beetle sister species. *Ecological Entomology, in review*.

Parker, E. S., Newton, I. L., & Moczek, A. P. (2020). (My Microbiome) Would Walk 10,000 miles: Maintenance and Turnover of Microbial Communities in Introduced Dung Beetles. *Microbial Ecology*, 1-12.

Renoz, F., Pons, I., & Hance, T. (2019). Evolutionary responses of mutualistic insect–bacterial symbioses in a world of fluctuating temperatures. *Current Opinion in Insect Science*, *35*, 20–26.

Roessner, E., Schoenfeld, J., & Ahrens, D. (2010). *Onthophagus* (Palaeonthophagus) medius (Kugelann, 1792)—a good western palaearctic species in the Onthophagus vacca complex (Coleoptera: Scarabaeidae: Scarabaeinae: Onthophagini). *Zootaxa*, 2629(1), 1-28.

Rosenberg, E., & Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome*, *6*(1), 78.

Roy, L., Bon, M. C., Cesarini, C., Serin, J., & Bonato, O. (2016). Pinpointing the level of isolation between two cryptic species sharing the same microhabitat: a case study with a scarabaeid species complex. *Zoologica Scripta*, 45(4), 407–420.

Shapira, M. (2016). Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. *Trends in Ecology and Evolution*, *xx*(7), 539–549.

Sharon, G., Segal, D., Ringo, J. M., Hefetx, A., Zilber-Rosenberg, I., & Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 110(12), 4852.

Silva, D. P., Vilela, B., Buzatto, B. A., Moczek, A. P., & Hortal, J. (2016). Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, 18(11), 3137–3148.

Sudakaran, S., Kost, C., & Kaltenpoth, M. (2017). Symbiont Acquisition and Replacement as a Source of Ecological Innovation. *Trends in Microbiology*.

APPENDIX

Developmental bias in horned dung beetles and its contributions to innovation, adaptation, and resilience

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REVIEW

WILEY

Developmental bias in horned dung beetles and its contributions to innovation, adaptation, and resilience

Yonggang Hu* | **David M. Linz*** | **Erik S. Parker*** | **Daniel B. Schwab*** | **Sofia Casasa** | **Anna L. M. Macagno** | **Armin P. Moczek**

Department of Biology, Indiana University, Bloomington, Indiana

Correspondence
Armin P. Moczek, Department of Biology, Indiana University, Bloomington, IN 47405.
Email: armin@indiana.edu

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Abstract

Developmental processes transduce diverse influences during phenotype formation, thereby biasing and structuring amount and type of phenotypic variation available for evolutionary processes to act on. The causes, extent, and consequences of this bias are subject to significant debate. Here we explore the role of developmental bias in contributing to organisms' ability to innovate, to adapt to novel or stressful conditions, and to generate well integrated, resilient phenotypes in the face of perturbations. We focus our inquiry on one taxon, the horned dung beetle genus *Onthophagus*, and review the role developmental bias might play across several levels of biological organization: (a) gene regulatory networks that pattern specific body regions; (b) plastic developmental mechanisms that coordinate body wide responses to changing environments and; (c) developmental symbioses and niche construction that enable organisms to build teams and to actively modify their own selective environments. We posit that across all these levels developmental bias shapes the way living systems innovate, adapt, and withstand stress, in ways that can alternately limit, bias, or facilitate developmental evolution. We conclude that the structuring contribution of developmental bias in evolution deserves further study to better understand why and how developmental evolution unfolds the way it does.

KEYWORDS

developmental symbiosis, doublesex, genetic accommodation, homology, insulin signaling, niche construction, Onthophagus, orthodenticle

1 | INTRODUCTION

Organismal form and function are generated by the processes of development, with some variants arising more readily than others, a phenomenon known as developmental bias (Uller, Moczek, Watson, Brakefield, & Laland, 2018). Such bias then structures amount and type of phenotypic variation available for evolutionary processes to act on. This biasing capacity of development is uncontroversial, as is the potential of developmental bias to limit, or constrain, adaptive evolution by preventing phenotypic variation from arising that would otherwise be favored by selection (Alberch, 1989; Arthur, 2004). What is controversial, however, is the creative role developmental bias may play in evolution by facilitating the production of novel, potentially adaptive variation (Laland et al., 2015). Similarly, it is now broadly understood that developmental bias is itself

*Yonggang Hu, David M. Linz, Erik S. Parker, and Daniel B. Schwab contributed equally to this work.

a product of developmental evolution shaped by past rounds of selection; thus, exactly how developmental bias affects developmental evolution may itself change over evolutionary time. Yet controversy surrounds the position that developmental bias may evolve such as to preferentially increase phenotypic variability in the direction favored by past natural selection (Moczek, 2012; Uller et al., 2018).

Here we explore the degree to which developmental bias facilitates adaptive evolution and evolvability by focusing on three dimensions critical to the developmental evolution of all living systems: their ability to generate novel variation (innovation), their ability to enhance the fit between organism and environment (adaptation), and their ability to withstand stress and perturbations (resilience). We reason that if developmental bias facilitates innovation, adaptation, and/or resilience, then theories and approaches in evolutionary biology will benefit from more explicit incorporations of developmental bias as a structuring force shaping the evolution of organismal form and function.

In the sections that follow we focus our analysis on the horned dung beetle genus *Onthophagus*, a model system in *evo devo* and *eco evo devo* (Choi et al., 2010; Kijimoto, Pespeni, Beckers, & Moczek, 2012; Moczek, 2009). We begin by examining the potential role of developmental bias at the level of gene regulatory networks in the origin of novel complex traits and their resilient integration within established trait complexes. Specifically, we assess the role of developmental bias in the origin and diversification of *Onthophagus* horns—exaggerated and highly diversified secondary sexual traits used in male combat over access to females (Moczek, 2005). In the second part, we explore if developmental bias may manifest in evolutionarily significant ways through developmental plasticity, that is, organisms' ability to respond to changes in their environment by adjusting aspects of their phenotypes. In particular, we explore if ancestral plasticity may bias the direction and speed of exotic *Onthophagus* populations' adaptations to novel or stressful conditions during the colonization of new habitats (Moczek, 2010). Lastly, we examine the potential significance of developmental bias emerging through host–symbiont interactions and niche construction. Specifically, we explore the role of interactions between *Onthophagus* hosts and their gut microbial symbionts and the systematic modification of environmental states in ways that have the potential to influence host development and diversification (Schwab, Casasa, & Moczek, 2019). Throughout we highlight promising future avenues to further assess the role of developmental bias in innovation, adaptation, and resilience, in *Onthophagus* horned dung beetles and beyond.

2 | DEVELOPMENTAL BIAS THROUGH GENE REGULATORY NETWORKS

Gene regulatory networks consist of the interactions between DNA sequences and their mRNA and protein products in a sequential-hierarchical fashion across developmental space and time (Carroll, Grenier, & Weatherbee, 2005; Davidson & Erwin, 2006). These interactions play critical roles in guiding the production and functional integration of biological form during development (Levine & Davidson, 2005), while changes in these interactions contribute significantly to the emergence of novel traits and trait functions in evolution (Ciliberti, Martin, & Wagner, 2007; Prud'homme, Gompel & Carroll, 2007). At the same time, the behavior of gene regulatory networks is inherently responsive to context (von Dassow, Meir, Munro, & Odell, 2000; Wagner, 2005). As a result, gene regulatory networks also contribute to the resilience of developmental processes and outcomes to perturbations arising from internal and external environmental influences. Thus, gene regulatory networks may be key sources of bias in the development and evolution of functional, resilient, and novel phenotypes (Payne, Moore, & Wagner, 2014; Uller et al., 2018).

The relationship between developmental bias at the level of gene regulatory networks and innovation may perhaps be most easily seen when developmental evolution repurposes pre-existing and preassembled networks to scaffold innovations (Hu et al., 2018; Linz, Hu, & Moczek, 2019; Shubin, Tabin, & Carroll, 2009; Wagner, 2014). In such cases, the direction, type, and functional integration of incipient innovations are shaped by the pre-existing configuration and system properties of repurposed gene networks (Tomoyasu, Ohde, & Clark-Hachtel, 2017; Wagner, 2007, 2014). *Onthophagus* horned beetles offer several valuable opportunities to explore the potential significance of bias through repurposing. For example, a long-standing research program has explored the origin of head horns, exaggerated secondary sexual traits used in competition over mates. Head horns are not modified versions of ancestral outgrowths or appendages, and are positioned on the dorsal head where insects or noninsect arthropods ancestrally never developed any type of projection (Grimaldi & Engel, 2005). Head horns are therefore neither homologous to other insect appendages, nor homonomous to other structures along the animal's body, thus fulfilling even the most stringent of definitions of evolutionary novelty (Wagner, 2014). Yet even though head horns constitute a relatively recent evolutionary invention, they found ways to integrate successfully within the dorsal head, itself an ancient trait

complex in existence ever since the origin of insects >420 MYA and whose embryonic assembly is governed by a gene network ultraconserved across phyla (Posnien, Schinko, Kittelmann, & Bucher, 2010). Recent work, therefore, aimed to explore the degree to which the repurposing of pre-existing, ancestral embryonic head patterning mechanisms may have been redeployed to facilitate the seamless integration of novel horns within the adult head.

Generally, the gene network that patterns the adult insect head is not well known. However, the network that patterns the same region during embryonic development is deeply conserved across taxa and well studied. Because adult heads derive through metamorphosis from their larval and embryonic precursors this embryonic head patterning network is thus a prime candidate for having been repurposed for both patterning the adult head and the integration of novelty therein. However, the larval head produced by embryonic patterning undergoes massive remodeling during the larval to adult metamorphic transition, obfuscating developmental and morphological correlations between stages. So while embryonic head patterning gene network components have well established spatial and temporal patterns of expression, it was initially unknown how these regions corresponded to adult head structures and in particular those that give rise to horns.

Using a unique larval fate-mapping approach, Busey, Zattara, and Moczek (2016) ablated concise larval head regions and assessed developmental defects produced in the adult head. This study established specific locations along the ocular-clypeoplabral boundary in the anterior presegmental region of the larval head as the corresponding tissue regions that give rise to posterior head horns in adult beetles, the most common position of head horns across *Onthophagus* beetles (Figure 1a–c). Once the developmental fate of these and other head regions was understood, candidate genes acting within and across region boundaries could then be functionally explored to assess their role in constructing and patterning adult morphology. For example, two transcription factors, *sine oculis 3*/*optix* (*six3*) and *orthodenticle* (*otd*) are expressed in complementary domains at the clypeolabral-ocular boundary across metazoan phyla during embryonic development (Li et al., 1996; Posnien, Koniszewski, Hein, & Bucher, 2011; Figure 1d). These two genes were thus key targets for further, postembryonic functional analysis which established a major role for *otd* in the formation and positioning of horns across *Onthophagus* species (Zattara, Busey, Linz, Tomoyasu, & Moczek, 2016). Upon downregulation of *otd*, horns were removed from typical horn-bearing regions, and instead formed ectopically in other normally non-horn-bearing regions (Figure 1e; Zattara et al., 2016). Importantly, this study also revealed that *otd* appears dormant, expressed but nonfunctional, in the dorsal heads of more basal hornless species such as *Tribolium,* while maintaining function during embryonic patterning. In contrast to *otd*, *six3*/*optix* was found to have no role in horn formation, even though it is critical for the embryonic head formation and must interact tightly with *otd* during this stage.

Combined, these data support the hypothesis that components of an ancient gene network already tasked with embryonic head development may have latent expression in adult head development, components of which can be reawakened and neofunctionalized to

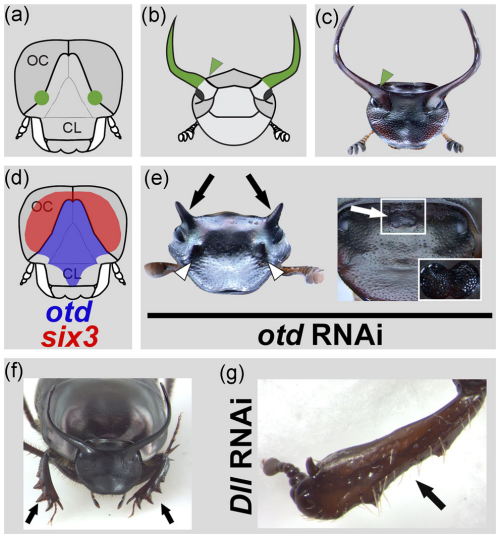

FIGURE 1 Developmental bias through gene regulatory networks exemplified by two classes of novelty in *Onthophagus* dung beetles. (a–c) Fate mapping approaches traced a specific location along the larval ocular-clypeolabral boundary (green dots in a) to adult posterior head horns (green region and green arrowheads in b and c). OC is the ocular region in the dark grey and CL is the clypeolabral region in light grey. (d) Two embryonic head patterning genes, *otd* (blue region) and *six3* (red region), have juxtaposed and mutually interdependent expression domains around the ocular-clypeolabral domain. (e) While *six3* RNAi causes no dorsal head defects, *otd* RNAi causes reduction of posterior horns (black arrows), induction of ectopic horns (white arrowheads) and induces a medial ectopic eye-like structure (white arrow and inset). (f) The tibial teeth of dung beetles (black arrows) are a modest novelty contained within the forelegs. (g) *Dll* RNAi causes severe disruptions in tibial teeth formation (black arrow) [Color figure can be viewed at wileyonlinelibrary.com]

integrate novelty within this region without compromising overall head patterning. Neofunctionalization can be accomplished in a way where the mutual interdependency of transcription factors at the embryonic stage can be shuffled or disengaged at the adult stage. The use of these genes, and the rewiring of the network they belong to, thus both facilitated novelty by providing customizable pre-existing mechanisms for spatial specification, but also biased positioning toward preferred locations, now reflected in the preponderance of posterior head horns across the *Onthophagus* phylogeny (Busey et al., 2016; Emlen, Corley Lavine, & Ewen-Campen, 2007).

Lastly, in an unexpected twist, *otd* downregulation, along with the major horn defects discussed above, also induced the formation of a medially-located ectopic eye-like structure, yet only in scarabaeid species (Figure 1e). In a second study, Zattara, Macagno, Busey, and Moczek (2017) further examined these eye-like structures revealing intact ommatidial lenses, crystalline cones, associated neural-like tissue within them as well as a transcriptomic landscape that mirrored that of regular compound eyes. In other words, these ectopic eyes appeared to be fully integrated morphologically and developmentally, yet their functionality was unknown. A behavioral assay was ultimately able to show that the ectopic eye was indeed photosensitive and fully functional, able to rescue a phototactic response in animals whose regular eyes had been surgically ablated. With the perturbation of horn formation and the simultaneous induction of functional eye-like structures, these results suggest that perturbing a gene network does not necessarily cause a region to disassemble. Instead knockdown of a single gene may allow the remaining gene network and associated developmental processes to reconfigure a morphological region in a functionally integrated manner, thereby highlighting how networks can contribute to resilience even when major network hubs are removed.

2.1 | Where does innovation start?

Beetle horns satisfy even the strictest definition of novelty —lacking homology or homonomy to other regions. At the same time, our understanding of the evolutionary process is rooted deeply within the notion of descent with modification—everything new must come from the old. Work on beetle horns (as well as evodevo generally) has now firmly established the significance of differential co-option and repurposing of gene networks as a common route to connect ancestral developmental features to novel morphological outcomes. Yet exactly how the former may yield the latter is far from understood: structures that fit strict novelty definitions (horns, eyes, butterfly eyespots, etc) are distinctly apomorphic, and as such their study has so far offered little insight into the earliest phases of innovation. Here, the study of structures that do not fully fit within the most stringent novelty definition may provide some clues.

One such example is the tibial teeth of dung beetles (Figure 1f). Tibial teeth typically consist of four-pointed projections along the outer margin of the tibia of the forelegs, which play a critical role in enhancing beetles' ability to dig into compact soil (Linz et al., 2019). On one side tibial teeth thus conveyed significant adaptive potential and facilitated scarab beetles' radiation into a novel ecological niche. On the other side, tibial teeth are fully contained within the tibia, and thus a leg segment whose homology status is unambiguous. Combined, tibial teeth, therefore, embody what might be considered an early, modest innovation. Recent work examined the gene networks that help instruct the formation of tibial teeth, and found, perhaps expectedly, that reuse and repurposing of genes and pathways that are locally available (such as genes ancestrally tasked with establishing the proximo-distal axis of the leg) dominated the developmental evolution of tibial teeth (Linz et al., 2019). In fact, the precise function of several locally available genes was often found to be recapitulated in their novel role: for instance the gene *Distalless* (*Dll*) is critical for establishing the P-D axis during development of the leg, and was also observed to execute a similar function specifically during the formation of tibial teeth (Figure 1g). Importantly, however, tibial teeth formation also turned out to rely on genes whose ancestral functions lie well outside a leg formation context: specifically, at least two genes well studied for their roles in embryonic patterning emerged as critical for proper tibial teeth formation, having acquired a function well outside their ancestral spatial and temporal domains.

Our results may suggest a model for how developmental evolution scaffolds innovation: first through the reuse of genes whose products are locally already available and whose ancestral functions are preadapted to support key aspects of the development of a given novel trait, followed by genes whose products ancestrally function completely outside the context of a given novel trait, and thus have to evolve both novel domains of expression, as well as new functions within this domain. Such a scenario would suggest that early innovation may be both *facilitated* by locally available developmental-genetic building blocks, providing immediate opportunities for diversification with relatively modest genetic changes, but also *biased* by the functional repertoire of exactly what genes and pathways may be available for repurposing and the developmental degrees of freedom they may provide.

2.2 | *Old functions for novel traits*: The integration of doublesex and insulin signaling in the evolution of sex- and nutrition-dependent development of head horns

Repurposing and associated biases are not restricted to the developmental evolution of morphological novelties, but also factor prominently in the *functional* diversification of novel traits. For example, as is common in the genus, *Onthophagus taurus* possesses an intersexual and intrasexual dimorphism in head horn development. Only males well-nourished during the larval stage grow into large adults with fully developed horns, nearly 10-fold longer than those of smaller males raised in suboptimal nutrition (Moczek, 1998), while all adult females regardless of nutritional conditions experienced as larvae develop a shallow ridge in the same head location. While head horns represent an evolutionary novelty, an extensive body of work now shows that the developmental mechanisms underlying their sex- and nutrition responsive growth have been recruited and repurposed from a diverse, ancestral regulatory toolbox.

First hints emerged through transcriptomic screens which provided a first comprehensive list of candidate genes putatively underlying the evolution and diversification of beetle horns (Choi et al., 2010; Kijimoto, Costello, Tang, Moczek, & Andrews, 2009). Among the many identified candidates the transcriptional expression of the ortholog of *Drosophila doublesex* (*dsx*) stood out. In *Drosophila*, sex-specific *dsx* isoforms regulate sexually dimorphic differentiation (Saccone, Salvemini, Pane, & Polito, 2008; Sánchez, Gorfinkiel, & Guerrero, 2001; Tanaka, Barmina, Sanders, Arbeitman, & Kopp, 2011), and orthologous sequences showed significant differential expression across male body regions and nutritional conditions in *Onthophagus*, suggesting a potential role of *dsx* in patterning intersexual but also possibly intrasexual horn dimorphisms. Investigations of *dsx* gene structure identified one male-specific isoform and at least five female-specific isoforms, as well as one non-sex-specific but likely function-less isoform (Kijimoto, Moczek, & Andrews, 2012). Subsequent functional assessments of these transcripts implicated the male isoform in the nutrition-dependent promotion of horns, whereas the female isoform(s) inhibited horn formation in females. Sex-specific *dsx* isoforms have since been shown to also promote and inhibit head horns in males and females of the rhinoceros beetle *Trypoxylus dichotomus* (Ito et al., 2013), and enhance and hinder nutrition-responsive growth of mandible in males and females of the stag beetles *Cyclommatus metallifer*, respectively (Gotoh et al., 2014). Collectively, these results suggest that by providing a pre-existing developmental switch mechanism responsive to somatic sex, the co-option of sex-specific *dsx* isoforms have repeatedly facilitated the sex-specific elaboration of horns and other weapons. However, how *dsx*-mediated horn expression became linked to nutrition was less clear. Here, recent work on the insulin signaling pathway has begun to provide important insights.

The insulin/insulin-like signaling pathway (IIS) is a highly conserved pathway well recognized for its role in regulating growth in response to nutrition across phyla (Barbieri, Bonafè, Franceschi, & Paolisso, 2003; Brogiolo et al., 2001). In insects, rich nutritional environments cause the insulin-producing cells (IPCs) in the brain to produce and secrete insulin-like peptides (ILPs) into the hemolymph. The ILPs bind to and activate the Insulin Receptor (InR) of the target tissues, which in turn activates a phosphokinase signal transduction cascade, thereby inducing cell growth and proliferation (Brogiolo et al., 2001; Géminard et al., 2006). Importantly, tissues differ in their sensitivity to IIS, resulting in different growth rates across tissues within an individual. For example, in *Drosophila*, wings and legs grow proportionally to body size in response to nutritional condition, while central nervous system and genitalia are much less sensitive to the nutritional state, resulting in minimal size variation even when nutritional conditions vary (Cheng et al., 2011; Koyama, Mendes, & Mirth, 2013; Shingleton, Das, Vinicius, & Stern, 2005; Tang, Smith-Caldas, Driscoll, Salhadar, & Shingleton, 2011). Studies on both rhinoceros beetles and *Onthophagus* horned beetles now also implicate the insulin signaling pathway as a critical transducer of nutritional conditions during the larval to pupal transition, including the relative growth of nutrition-sensitive horns and nutrition-insensitive genitalia (Casasa & Moczek, 2018a; Emlen, Warren, Johns, Dworkin, & Lavine, 2012). Importantly, both rhinoceros beetles (subfamily Dynastinae) and *Onthophagus* dung beetles (subfamily Scarabaeinae) are believed to represent independent inventions and radiations of sexually dimorphic and exaggerated horns (Emlen et al., 2007). While both lineages appear to have relied on the co-option of the IIS, data available to date suggest that key regulatory functions are carried out by different pathway members in the two subfamilies: In *Trypoxylus* rhinoceros beetles, downregulation of the insulin receptor InR reduces male horn length but leaves genitalia unaffected (Emlen et al., 2012). Conversely, in *Onthophagus*, the same manipulation has no effect on the body size—horn size allometry, but significantly reduces genitalia size relative to body size (Casasa & Moczek, 2018a). Here, however, knockdown of Fork head, subgroup O (Foxo, a growth suppressor downstream of the InR) greatly increases head horn length in small, low-nutrition males,

while modestly decreasing it in large, high-nutrition males, thereby linearizing the normally sigmodal body size—horn size allometry. At the same time, *Foxo*^{RNAi} also increases nutrition sensitivity of genitalia. Most importantly, Casasa and Moczek (2018a) provided the first evidence suggesting a functional link between *dsx* expression (see above) and insulin signaling by demonstrating that *dsx* expression significantly decreases following knockdown of InR (Casasa & Moczek, 2018a). Taken together, these results suggest that by co-opting the IIS pathway horn formation acquired the ability to become exquisitely nutrition-responsive. Furthermore, by then linking *dsx* expression to IIS signaling, horns evolved the ability to exhibit nutrition responsive growth in a strictly sex-specific manner, thereby setting the stage for the dramatic radiation in sexual dimorphisms and male polyphenisms of this genus. More generally, these results suggest once again that morphological innovation and diversification are facilitated but also biased by the developmental opportunities and limits that emerge when a pre-existing developmental tool kit is reimplemented over and over again. Yet at the same time, by evolving novel interactions between pre-existing components of said toolkit, additional developmental degrees of freedom are generated with which evolution can subsequently tinker.

3 | DEVELOPMENTAL BIAS THROUGH DEVELOPMENTAL PLASTICITY

Developmental or *phenotypic plasticity* refers to a developing organism's ability to alter aspects of phenotype expression in response to changes in environmental conditions. Such responses may be subtle or dramatic, reversible or not, and can be shaped by either long periods of prior selection due to recurring or predictable environmental fluctuations, or alternatively, by conditions encountered for the very first time (Moczek, 2009). In all of these situations, developmental plasticity has the potential to exert developmental bias on variation in phenotype expression visible to selection, thereby shaping subsequent evolutionary trajectories (West-Eberhard, 2003).

For example, developmental plasticity is well established as a mechanism enabling organisms to maintain high fitness in the face of fluctuating environments, and in such cases may buffer the effects of diversifying selection, thereby limiting adaptive radiations (Schlichting & Pigliucci, 1998). In contrast, plasticity may facilitate rapid phenotypic divergences when populations colonize novel habitats or encounter major environmental perturbations (Hendry, 2016; Yeh & Price, 2004). Such immediate plasticity-mediated responses in development may be further enhanced through the process of *phenotypic accommodation*, that is, the adaptive mutual adjustment of variable aspects of the phenotype during development, occurring without any genetic change (West-Eberhard, 1998). For example, when *Polypterus* fish are forced to develop in an environment in which they have to walk on their pectoral fins more than swim, fish develop a more efficient gait during their lifetimes, accompanied by bone structure and musculature changes more suited to a terrestrial, walking lifestyle (Standen, Du, & Larsson, 2014). All these phenotypic adjustments improve trait integration and performance within a novel, stressful environment. Furthermore, while these changes manifest within a single generation in the absence of genetic changes, they nevertheless parallel some of the same changes observed in the fossil record during the water-to-land transition of tetrapods.

One mechanism that may enable plastic responses to precede and bias subsequent genetic evolution is *genetic accommodation*. Genetic accommodation is broadly defined as a change in gene frequency due to selection on the regulation of an environmentally-induced response (West-Eberhard, 2003). As such it constitutes a mechanism whereby initially environmentally induced traits, including the products of phenotypic accommodation, may become genetically stabilized or canalized, for instance when plastic responses to environmental conditions make visible to selection cryptic genetic variation accumulated during previous generations (Paaby & Rockman, 2014). Evidence in support of genetic accommodation derived initially from environmental perturbation and artificial selection experiments (*Drosophila*: Dworkin, 2005; Rutherford & Lindquist, 1998; Waddington, 1953; *Manduca sexta*: Suzuki & Nijhout, 2006; *Caenorhabditis*: Sikkink, Reynolds, Ituarte, Cresko, & Phillips, 2014; *Arabidopsis*: Queitsch, Sangster, & Lindquist, 2002; fungi: Cowen & Lindquist, 2005; cyanobacteria: Walworth, Lee, Fu, Hutchins, & Webb, 2016). More recently, a growing number of studies have shown genetic accommodation in natural populations (spadefoot toad tadpoles: Gomez-Mestre & Buchholz, 2006; Ledón-Rettig, Pfennig, & Nascone-Yoder, 2008; Levis, Isdaner, & Pfennig, 2018; threespine sticklebacks: Robinson, 2013; Shaw, Scotti, & Foster, 2007; Wund, Baker, Clancy, Golub, & Foster, 2008; *Daphnia*: Scoville & Pfrender, 2010; house finches, Badyaev, 2009; Badyaev, Potticary, & Morrison, 2017; cavefish: Rohner et al., 2013). Collectively, this body of work demonstrates the feasibility and potential significance of genetic accommodation in evolution. Moreover, it highlights the potential for developmental bias, via environmentally

induced phenotypes, in the evolution of adaptive traits. Nonetheless, several critical dimensions remain to be addressed. For example, exactly how fast evolution by genetic accommodation may contribute to diversification, and the extent to which it actually does so in natural populations, remain largely unclear. Similarly, earlier work posited that because behavioral traits often exhibit both extreme plasticity and evolutionary lability, behavior may be more likely to evolve by genetic accommodation than other organismal features such as morphology (Allf, Durst, & Pfennig, 2016; West-Eberhard, 1986 &, 2003). However, little comparative work has addressed this issue thus far.

Onthophagus taurus has emerged as a promising study system to advance these and related questions, in part due to the existence of recently established and rapidly diverging exotic populations (reviewed in Casasa & Moczek, 2018b). While originally restricted to its native Mediterranean distribution, in the 1970s this species was introduced to Eastern and Western Australia to help control cow dung and dung-breeding flies (Tyndale-Biscoe, 1996) as well as to the Eastern United States by accident (Fincher & Woodruff, 1975). Since introduction, both Eastern US and Western Australian populations have diverged rapidly in diverse traits, both from each other and relative to their Mediterranean source population. This differentiation has likely been the product of differential adaptations to local dung beetle densities (very high in Western Australia, low in the Eastern US) and the resulting divergent intensity of mate and resource competition (Moczek, 2003; Figure 2a), as well as an expansion of the Eastern US population into a colder and more humid climatic niche (Silva, Vilela, Buzatto, Moczek, & Hortal, 2016). Trait differences between populations are maintained in common garden conditions, and include morphology (e.g., adult body size, allometric threshold for horn induction, male genitalia shape, female fore tibia shape), development and physiology (e.g., degree and timing of sensitivity to juvenile hormone, sensitivity to serotonin upregulation, duration of larval development, developmental responses to temperature stress), and behavioral and life-history traits (provisioning behavior and fitness; Beckers, Anderson, & Moczek, 2015; Macagno, Beckers, & Moczek, 2015; Macagno et al., 2011; Macagno, Moczek, & Pizzo, 2016; Macagno, Zattara, Ezeakudo, Moczek, & Ledón-Rettig, 2018; Moczek & Nijhout, 2002 &, 2003; Moczek, Hunt, Emlen, & Simmons, 2002; Newsom, Moczek, & Schwab, in review).

Considering six of these traits (body size and horn allometry threshold for morphology; brood ball mass and nesting depth for behavior; and brood ball number and eclosion success for life history), a recent study by Casasa and Moczek (2018b) examined the presence and direction of plasticity in response to variation in adult density in the Mediterranean source population. In controlled lab conditions, native beetles were subject to either very high (Western Australia-like) or very low (Eastern US-like) adult densities, and just 3 weeks of this treatment were sufficient to induce measurable plasticity in four of the six traits studied. Average responses matched the direction of canalized differences between descendent exotic populations in one morphological trait (adult body size) and one life-history trait (fecundity, as measured by the number of brood balls produced; Figure 2b). For these two traits, results are consistent with a "plasticity first" scenario, whereby plastic responses to environmental conditions unveil phenotypic variation that is later canalized by selection (Levis & Pfennig, 2016). Two other traits (one behavioral—the amount of food provisioned to offspring, and one life-history trait—eclosion success) exhibited plasticity in the direction *opposite* to that predicted based on the differences between exotic populations. However, by itself this observation does not reject the possibility of a "plasticity first" scenario: while plasticity in response to a novel environment is assumed to not be able to anticipate adaptive variation (Moczek, 2007), to *facilitate* adaptive evolution, it is only necessary that variation among ancestral reaction norms *encompasses at least some* novel variants that selection can promote (Casasa & Moczek, 2018b; Figure 2c).

Studies such as these suggest that developmental plasticity may bias evolution already at the very earliest stages of population differentiation, and possibly across trait categories, through the environment-responsive production of functionally integrated and potentially adaptive phenotypes. However, to determine whether these results are indeed generalizable will require the study of many more and diverse taxa, traits, and potentially inductive environmental contexts. Studies are also needed to better understand the molecular, genetic, and transcriptomic mechanisms of genetic accommodation. For example, recent work by Levis et al. (2018) examined the role of gene expression plasticity in spadefoot toads. In the genus *Spea,* a diet-induced polyphenism results in either omnivorous or carnivorous tadpole morphologies. Using a closely related nonpolyphenic species as a proxy for the ancestral condition, and exposing this species to a novel carnivorous diet, Levis et al. (2018) were able to document gene expression plasticity in genes associated with polyphenic development. This study marks an important effort to assess the mechanistic basis of genetic accommodation in the wild, but it does so only for relatively few genes. Yet, development of complex traits such as alternative feeding morphs (*Spea*) or male reproductive morphs (*Onthophagus*) is likely underpinned by hundreds or thousands of genes, which will ultimately necessitate a much broader genome- and transcriptome-

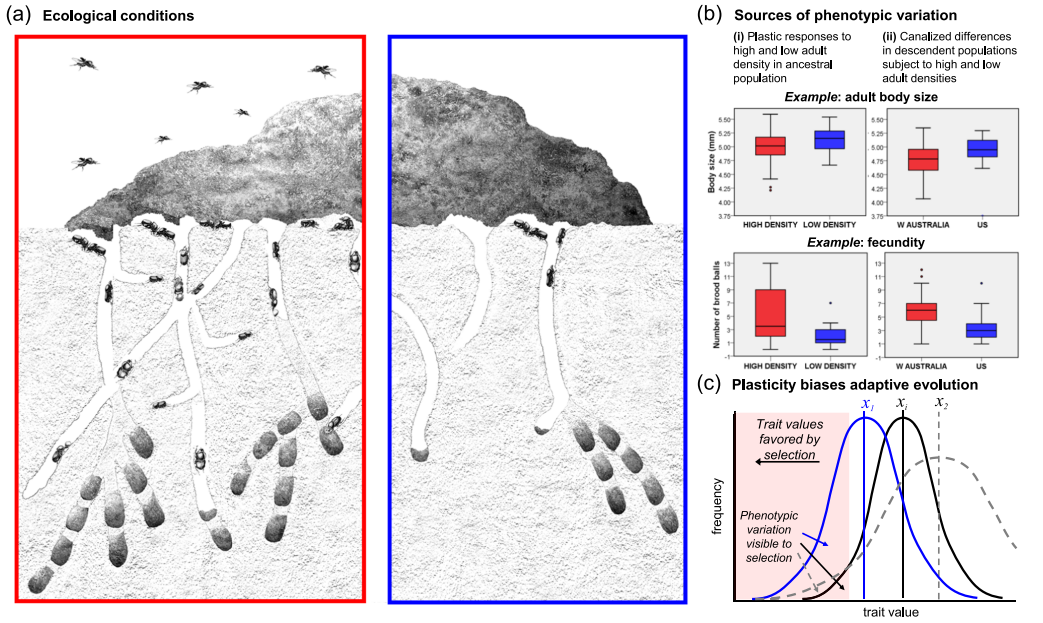

FIGURE 2 Developmental bias through developmental plasticity. (a) The horned dung beetle species *Onthophagus taurus* is subject to highly disparate ecological and social conditions in two exotic ranges. In Western Australia (WA, left, red frame) hundreds to thousands of individuals compete for breeding opportunities while in the Eastern United States (US, right, blue frame) local densities are up to three orders of magnitude lower and mate and resource competition are relaxed (drawing by Barret Klein). Since establishment WA and US populations have diverged heritably in a suite of behavioral, physiological, developmental, and morphological traits. (b) Developmental plasticity yields changes in phenotype expression that parallel canalized differences between populations. Plastic responses in beetles derived from an ancestral Mediterranean population to experimentally controlled high (red) or low (blue) densities yield significant phenotypic differences that parallel both direction and magnitude of evolved, canalized differences between high density Western Australian (WA, red) and low density Eastern US (US, blue) populations (data are from Beckers et al., 2015 and Casasa & Moczek, 2018b). (c) Plasticity biases evolution by altering type and frequency of phenotypic variation visible to selection. Plastic responses may bias adaptive evolution by shifting the initial mean (x_1) and frequency distribution of phenotypic variation in a direction favored by selection (x_1). Plasticity may bias adaptive evolution even in cases in which the mean phenotype shifts away from trait values favored by selection (x_2) as long as variation among ancestral reaction norms encompasses at least some novel variants that selection can promote [Color figure can be viewed at wileyonlinelibrary.com]

wide perspective (e.g., Ghalambor et al., 2015). A more comprehensive understanding of the mechanistic basis of genetic accommodation will likely enable key insights into how environmentally sensitive gene regulatory networks are rewired to produce integrated and functional phenotypes that have the potential to influence evolutionary trajectories.

4 | DEVELOPMENTAL BIAS THROUGH SYMBIOSES AND NICHE CONSTRUCTION

Traditionally, *evo devo* biologists have sought to explain biased patterns of phenotypic variability by interrogating the endogenous gene regulatory, physiological, and developmental mechanisms that regulate morphogenesis (Arthur, 2004; Uller et al., 2018). In the preceding sections, we focused on these same levels of biological organization, and explored how evolutionary processes may be biased towards deploying the same pre-existing and preassembled genes and gene networks in the advent of novel structures (Linz et al., 2019; Shubin et al., 2009), and discussed how environment-responsive development may be primed to generate well-integrated, functional, and sometimes adaptive variants in the face of ecological stressors (Casasa & Moczek, 2018b). Yet, bias may manifest at additional and even extra-organismal dimensions, for example when organisms actively modify their own selective environments through the process of *niche construction* or by engaging in *developmental symbioses* that can structure important functional variation. Here, we suggest that these

processes may both independently and synergistically play fundamental roles in promoting normal development in *Onthophagus* beetles and facilitate the formation of well-integrated, resilient phenotypes in the face of environmental perturbations.

4.1 | Niche construction, symbiosis, and the reciprocal nature of development

Niche construction occurs when organisms, via their physiology and behaviors, modify their own and each other's niches in systematic ways (Odling-Smee, Laland, & Feldman, 2003). When directly modifying developmental environments, the nature and scope of these modifications can range from the production of physical structures such as burrows or pupation chambers, to alterations of chemical states in the surrounding environment. One common and adaptive function of these modifications is to lend resilience to organisms developing under challenging environmental conditions. Among insects, some of the most prominent examples of this form of niche construction include gall-forming flies and tent building caterpillars, whose physical constructions buffer them from predators, parasites, and thermal fluctuations (Abrahamson, Sattler, McCrea, & Weis, 1989; Joos, Casey, Fitzgerald, & Buttemer, 1988). For these and many other organisms, niche construction is a characteristic feature of normal development, enhancing the fit between organism and environment (Laland, Odling-Smee & Gilbert, 2008; Schwab & Moczek, 2017). When scaled-up from an individual organism's development to the level of populations, evolutionary models suggest that niche construction can significantly alter the rate and direction of evolution and influence which genetic variants are maintained or lost (Kylafis & Loreau, 2008; Laland, Odling-Smee, & Feldman, 1999). Importantly, niche construction is fully consistent with phenomena that have been historically studied under alternative frameworks. For instance, parental effects may be considered a form of niche construction in which parents construct developmental environments such as nests or brood chambers for offspring, and ecosystem engineering may represent a form of niche construction expressed at the level of communities and beyond (Day, Laland, & Odling-Smee, 2003). In each case, niche construction represents a potent form of reciprocity between organism and environment that has the potential to shape patterns of phenotypic variation. Yet this reciprocity does not need to end at the boundaries of the individual organism, and a growing body of work illustrates that reciprocal niche construction between multiple organisms and their shared environmental domains can profoundly affect both development and evolution of phenotypic variation (Chiu & Gilbert, 2015).

The significance of reciprocal niche construction in developmental evolution is perhaps best illustrated by the rapidly growing work on host–microbe interactions. Recent technological advances in the ability to taxonomically characterize and evaluate the potential functions of microbial communities of eukaryotic hosts has resulted in a far greater appreciation of the importance of microbes for virtually all aspects of host biology, including in the regulation of the normal development of their hosts (M. McFall-Ngai et al., 2013). Indeed, the influence of microbial symbionts can be observed across all stages of animal development. For instance, species of obligate intracellular bacteria promote germline proliferation in nematodes (*Wolbachia*: Foray, Pérez-Jiménez, Fattouh, & Landmann, 2018), and protect embryos against pathogenic infections in arthropods (*Wolbachia* and *Spiroplasma*: Jaenike, Unckless, Cockburn, Boelio, & Perlman, 2010; Teixeira, Ferreira, & Ashburner, 2008). During postembryonic development, microbial symbionts have been implicated in instructing the completion of digestive (e.g., guts of mice: Hooper & Gordon, 2001; Sommer & Bäckhed, 2013; and zebrafish: Bates et al., 2006; Rawls, Samuel, & Gordon, 2004) and immune system development (reviewed in Gilbert, Bosch, & Ledón-Rettig, 2015) across vertebrate taxa. Furthermore, microbial symbionts have been linked to transitions between developmental stages, producing signals that induce metamorphosis in a suite of marine invertebrate taxa such as tubeworms, corals, and sponges (Shikuma et al., 2014; Sneed, Sharp, Ritchie, & Paul, 2014; Whalan & Webster, 2014). These developmental symbioses also have the potential to be highly reciprocal. For example, bacterially-mediated induction of light organ formation in the bobtail squid, in turn, activates gene expression changes in the inducing bacteria, causing the bacteria to express the bioluminescent properties that are characteristic of the new organ (M. J. McFall-Ngai, 2014). In many instances, these developmentally significant symbionts are passed down or selectively acquired from host environments during development, ensuring the maintenance of their functions across host generations. Alongside niche construction, developmental symbioses thus present another avenue through which organisms reciprocally interact to facilitate each others' as well as their own development (see Gilbert, 2019, this issue, for additional examples).

Although niche construction and developmental symbiosis have emerged from disparate conceptual frameworks and empirical investigations, these processes share a common potential to bias the outcomes of development and developmental evolution (Laland et al., 2015). For instance, developmental bias is an inherent feature of niche construction, in which organisms engage with and predictably alter their environments in ways that may better suit their traits (Schwab & Moczek, 2017). In so

doing, organisms bias their selective environment while simultaneously channeling the expression of developmental variation toward particular states. The latter is best demonstrated when niche constructors plastically respond to the environments that they themselves have generated (see below; Schwab, Casasa, & Moczek, 2017). These modifications can lead to further, transgenerational bias when altered environmental states are inherited, including via the inheritance of microbial symbionts that are necessary for normal development. In developmental symbiosis, bias is expressed not only through the effects that host–microbe interactions have on the production of functional variation, but also in how these interactions can bias or facilitate innovation in the face of novel environments. Yet, while it is true that both niche construction and developmental symbioses present important sources of developmental bias, few systems have been leveraged to experimentally evaluate the potential individual and synergistic contributions of each process to phenotypic and evolutionary outcomes. For instance, although niche construction is thought to play important roles in the development and evolution of niche constructors, their descendants, and even other species, few experimentally tractable model systems have been developed in which the mechanisms of niche construction (a) are well understood, (b) can be experimentally manipulated, and (c) produce effects that can be rigorously quantified. Conversely, while the experimental study of the causes and consequences host–microbe interactions have a long and productive history, additional systems are needed to fully evaluate the role of developmental symbiosis in e.g., rapid adaptation to local environments, ecological radiations, or speciation.

4.2 | Developmental symbiosis: A characteristic feature of *Onthophagus* life history, growth, and survival

The life cycle of *Onthophagus* dung beetles provides a promising, experimentally tractable model system in which to address these questions as both niche construction and symbiosis play critical roles in facilitating normal development (Figure 3a). These contributions first begin when mothers engage in niche construction by digging deep tunnels underneath cow dung pats, within which they construct brood balls. When mothers invest in burying brood balls deep underground, this tunneling behavior provides developing offspring with a stable thermal niche (Snell-Rood, Burger, Hutton, & Moczek, 2016) and increased access to oxygen (Schwab, Flores, Linz, Moczek, & Tennessen, in prep), while brood ball construction provides each larva with all the food needed to complete development and metamorphosis. Moreover, each brood ball is further endowed with a maternal fecal pedestal onto which a single egg is oviposited (Estes et al., 2013). Upon hatching, larvae immediately consume this pedestal, thereby inoculating themselves with maternal gut microbiota (Schwab, Riggs, Newton, & Moczek, 2016).

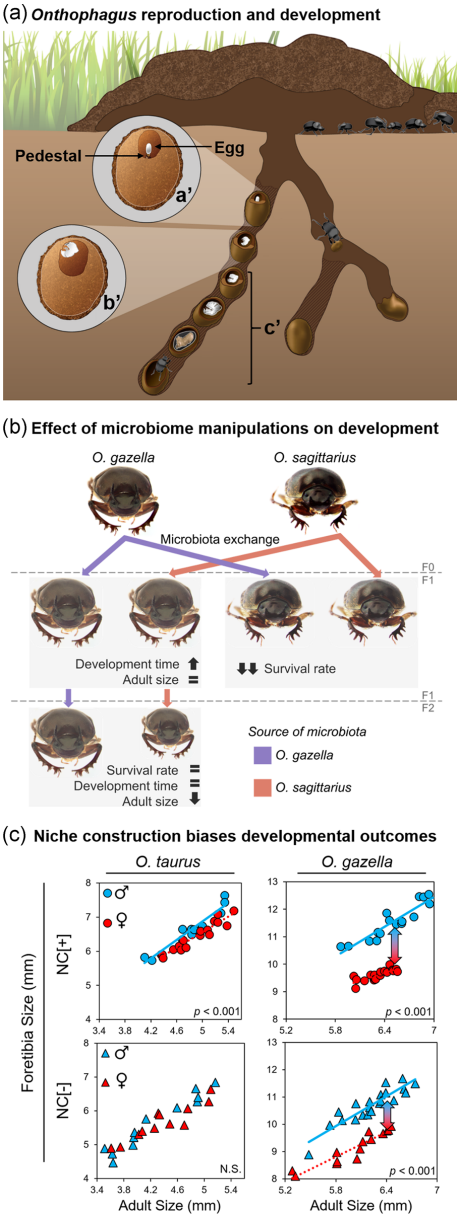

FIGURE 3 Continued

Microbes have long been hypothesized to play a critical role in enabling both juvenile and adult *Onthophagus* to subsist and diversify upon dung, which is primarily composed of complex polysaccharides such as cellulose and relatively low in amino acids (Flachowsky & Hennig, 1990; Frank, Brückner, Hilpert, Heethoff, & Blüthgen, 2017; Halffter & Edmonds, 1982; Holter, 2016; Muller, 1980). Recent work on *O. taurus* and the closely related genus *Euoniticellus* now shows that pedestal microbiota are enriched for genes implicated in cellulose degradation and nitrogen fixation (Estes et al., 2013; Shukla, Sanders, Byrne, & Pierce, 2016). Additional experimental support for this hypothesis derives from the demonstration that *Onthophagus* larvae forced to develop without their pedestal microbiota require more time to complete development and metamorphose into smaller adults compared to larvae that are provided with their pedestal microbes. Importantly, these disparities are further exaggerated under ecologically relevant temperature and desiccation stressors (Schwab et al., 2016). Furthermore, pedestal microbiota conveys resilience against dung-associated pathogens: larval mortality in the presence of the entomopathogenic fungus, *Metarhizium anisopliae*, increases by 20–40% when reared in the absence of pedestal microbes (Schwab et al., in prep). Lastly, host species appear to have specialized onto nonequivalent sets of microbial partners. Specifically, recent work shows that the exchange of pedestals between two dung beetle species results in pronounced negative survival outcomes for one host species, while the other species demonstrates modest developmental delays with no significant effect on survival (Figure 3b). These findings provide the first experimental evidence that different *Onthophagus* host species may diverge in the extent to which they rely on gut microbiota to support normal development (Parker et al., 2018).

FIGURE 3 Developmental bias through symbiosis and niche construction. (a) *Onthophagus* mothers engage in niche construction by creating subterranean brood balls made of dung. In addition to providing all the dung that offspring will need to complete their development, mothers deposit a fecal secretion containing gut microbiota known as the pedestal, upon which they lay a single egg (a'). Immediately following hatching, larvae consume the pedestal and begin engaging in niche constructing behaviors (b'), which will continue through pupation and into adulthood (c') (image modified after Estes et al., 2013). (b) The exchange of the maternally-transmitted pedestal microbiota between two dung beetle species results in developmental delay and increased mortality compared with beetles receiving their own microbiota (Parker, Dury, & Moczek, 2018). The magnitude of the negative effects uncovered through pedestal exchange differed between species, and in the case of one species, these effects were inherited across generations. (c) Larvae that engage in niche construction during normal development (NC[+]) express a significant sexual dimorphism in traits such as the foretibia, with males of *O. taurus* and *O. gazella* developing longer foretibia than females. When niche construction is experimentally inhibited (NC[−]), this dimorphism is either eliminated (*O. taurus*) or significantly reduced in magnitude (*O. gazella*; image modified after Schwab et al., 2017) [Color figure can be viewed at wileyonlinelibrary.com]

4.3 | Niche construction as a critical and evolvable feature of normal development

Shortly after feeding on the maternally-provisioned pedestal, larvae begin expressing a range of putative niche constructing behaviors that continue throughout their development. For instance, larvae mechanically manipulate surrounding dung to alter the physical composition of the brood ball throughout their growth period, repairing the brood ball where maternal construction is inadequate, and eventually constructing a complex pupation chamber from dung fibers and the beetle's own feces shortly before the metamorphic molt. Throughout this time, larvae defecate throughout their brood ball, thereby distributing pedestal-derived microbiota across the brood ball microenvironment, and then refeed on their own excrement until metamorphosis (Schwab et al., 2017). Recent experimental studies suggest that these collective modifications may further bias or promote particular developmental and fitness outcomes. For instance, experimentally suppressing the extent to which larvae can directly modify their brood ball environment decreases growth and common proxies of fitness (i.e., brood ball size and number produced) in multiple species of *Onthophagus*. Furthermore, suppressing niche construction alters scaling relationships in a number of key morphological traits, and even eliminates or substantially diminishes the degree of sexual dimorphism between male and female tibiae (Figure 3c; Schwab et al., 2017). Although the contribution of individual larval behaviors to these niche construction phenotypes is still unclear, preliminary findings suggest that the spreading of larval feces throughout the brood ball may generate a symbiont-mediated external rumen that aids in the chemical breakdown of chitin, lignin, and cellulose, thus promoting larval growth by making more readily digestable carbohydrates available to larvae (Schwab et al., 2017). Intriguingly, the developmental consequences of larval niche construction may coevolve with those of maternal niche construction: females derived from the Eastern US population of *O. taurus*, which engage in high levels of maternal care as measured by their deep brood ball burial depth, produce offspring

that exhibit a pronounced increase in development time when this maternal niche construction is inhibited, doing so regardless of the presence or absence of larval niche construction. However, in the relatively low maternal care Western Australia population, development time increases *only* when larval niche construction is inhibited, doing so regardless of the presence or absence of maternal niche construction. These results suggest that larvae from the Western Australia population may have undergone selection to compensate for low maternal niche construction by increasing investment in, and thereby their reliance upon, larval niche construction (Dury, Moczek, & Schwab, in review). Altogether, these early experimental findings suggest that niche construction is a critical *and evolvable* component of environmentally-responsive development in *Onthophagus*.

Although studies of *Onthophagus* niche construction and developmental symbiosis are only in their early stages, the findings presented here suggest that both of these highly reciprocal processes play fundamental roles in supporting normal development. It is clear, for instance, that both processes are capable of biasing the nature of phenotypic variation that results from ontogeny, that they lend resilience to development in the presence of ecological challenges, and that their effects are evolvable at the level of populations and species. Yet much remains to be explored. Of particular interest is determining what role, if any, the microbiota plays in the adaptation of *Onthophagus* beetles to novel environments. As discussed above, diverse *Onthophagus* species have been introduced around the world, and some of these introductions, such as that of *O. taurus* into the Eastern United States, have resulted in remarkable climatic niche expansions (Silva et al., 2016). Work exploring how developmental bias may have enabled such range expansions, whether acting through host-symbiont interactions, niche construction, or developmental plasticity, is currently ongoing. More generally, experimental studies of niche construction must expand to additional model systems beyond *Onthophagus* to garner a more complete understanding of the developmental and evolutionary consequences of this process in natural populations.

5 | CURRENT FRONTIERS IN THE STUDY OF DEVELOPMENTAL BIAS

In this review, we sought to explore the role of developmental bias across diverse levels of biological organization in the genesis of novel, adaptive, and resilient phenotypes within a single taxon, the horned dung beetle genus *Onthophagus*. We find developmental bias to be pervasive, able to shape patterns of phenotypic variation across diverse traits, and able to bias evolutionary changes over both macro- and micro-evolutionary time scales. Our findings thus add to a growing call to investigate the role of developmental bias in evolution more systematically and across a broader array of taxa, traits, and environmental contexts.

Our discussion also highlights several areas of particular significance. For example, because developmental bias is itself a product of evolution shaped by past rounds of selection, how developmental bias affects evolution may change over evolutionary time. The studies highlighted here broadly support this notion, and do so across disparate evolutionary time scales. For example, the deeply conserved head-patterning mechanisms that evolved in pre-Cambrian times now bias head innovations in derived insect lineages, while host–microbiome interactions that most likely originated when scarab beetles first evolved a dung-feeding life style (perhaps as recent as 50 MYA: Sole & Scholtz, 2010) now shape divergences among recently evolved *Onthophagus* species. Yet exactly how fast developmental bias can evolve, and the direction of this evolution, remain understudied. An especially contentious perspective derives from the hypothesis that developmental bias may evolve such as to preferentially modify phenotypic variability in the direction favored by past natural selection (Uller et al., 2018). The studies reviewed here suggest that developmental biases resulting from ancestral plasticity, developmental symbioses, and niche construction may well evolve in ways consistent with such a scenario, but more direct tests are needed to evaluate this hypothesis. Another poorly explored frontier concerns the existence and possible consequences of *interactions* among different types of developmental bias. Work on *Onthophagus* has begun to hint at a possibly significant synergism between developmental symbioses and niche construction (Schwab et al., 2017). Yet much more work is needed to evaluate if and how different types of developmental bias, operating on different levels of biological organization, interact in ways that may either restrain, combine, or synergize their impact on developmental evolution. Combined, such investigations into the role of developmental bias in evolution have the potential to significantly enhance our understanding of why and how organismal evolution unfolds the way it does.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Yonggang Hu http://orcid.org/0000-0002-3438-7296
David M. Linz http://orcid.org/0000-0003-2096-3958
Erik S. Parker http://orcid.org/0000-0002-5537-7900
Daniel B. Schwab http://orcid.org/0000-0001-5094-2598
Sofia Casasa http://orcid.org/0000-0002-5910-3739
Anna L. M. Macagno http://orcid.org/0000-0003-4133-2098

REFERENCES

Abrahamson, W. G., Sattler, J. F., McCrea, K. D., & Weis, A. E. (1989). Variation in selection pressures on the goldenrod gall fly and the competitive interactions of its natural enemies. *Oecologia*, *79*, 15–22.

Alberch, P. (1989). The logic of monsters: Evidence for internal constraint in development and evolution. *Geobios*, *22*, 21–57.

Allf, B. C., Durst, P. A. P., & Pfennig, D. W. (2016). Behavioral plasticity and the origins of novelty: The evolution of the rattlesnake rattle. *The American Naturalist*, *188*, 475–483.

Arthur, W. (2004). The effect of development on the direction of evolution: Toward a twenty-first century consensus. *Evolution & Development*, *6*, 282–288.

Badyaev, A. V. (2009). Evolutionary significance of phenotypic accommodation in novel environments: An empirical test of the Baldwin effect. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *364*, 1125–1141.

Badyaev, A. V., Potticary, A. L., & Morrison, E. S. (2017). Most colorful example of genetic assimilation? Exploring the evolutionary destiny of recurrent phenotypic accommodation. *The American Naturalist*, *190*, 266–280.

Barbieri, M., Bonafè, M., Franceschi, C., & Paolisso, G. (2003). Insulin/IGF-I-signaling pathway: An evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of Physiology-Endocrinology and Metabolism*, *285*, E1064–E1071.

Bates, J. M., Mittge, E., Kuhlman, J., Baden, K. N., Cheesman, S. E., & Guillemin, K. (2006). Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental Biology*, *297*, 374–386.

Beckers, O. M., Anderson, W., & Moczek, A. P. (2015). A combination of developmental plasticity, parental effects, and genetic differentiation mediates divergences in life history traits between dung beetle populations. *Evolution & Development*, *17*, 148–159.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., & Hafen, E. (2001). An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology*, *11*, 213–221.

Busey, H. A., Zattara, E. E., & Moczek, A. P. (2016). Conservation, innovation, and bias: Embryonic segment boundaries position posterior, but not anterior, head horns in adult beetles. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *326*, 271–279.

Carroll, S. B., Grenier, J. K., & Weatherbee, S. D. (2005). *From DNA to diversity: Molecular genetics and the evolution of animal design*. Oxford, MA: Blackwell Science Ltd.

Casasa, S., & Moczek, A. P. (2018a). Insulin signalling's role in mediating tissue-specific nutritional plasticity and robustness in the horn-polyphenic beetle Onthophagus taurus. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *285*, 20181631.

Casasa, S., & Moczek, A. P. (2018b). The role of ancestral phenotypic plasticity in evolutionary diversification: Population density effects in horned beetles. *Animal Behaviour*, *137*, 53–61.

Cheng, L. Y., Bailey, A. P., Leevers, S. J., Ragan, T. J., Driscoll, P. C., & Gould, A. P. (2011). Anaplastic lymphoma kinase spares organ growth during nutrient restriction in *Drosophila*. *Cell*, *146*, 435–447.

Chiu, L., & Gilbert, S. F. (2015). The birth of the holobiont: Multispecies birthing through mutual scaffolding and niche construction. *Biosemiotics*, *8*, 191–210.

Choi, J.-H., Kijimoto, T., Snell-Rood, E., Tae, H., Yang, Y., Moczek, A. P., & Andrews, J. (2010). Gene discovery in the horned beetle *Onthophagus taurus*. *BMC Genomics*, *11*, 703.

Ciliberti, S., Martin, O. C., & Wagner, A. (2007). Innovation and robustness in complex regulatory gene networks. *Proceedings of the National Academy of Sciences United States of America*, *104*, 13591–13596.

Cowen, L. E. (2005). Hsp90 potentiates the rapid evolution of new traits: Drug resistance in diverse fungi. *Science*, *309*, 2185–2189.

Davidson, E. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science*, *311*, 796–800.

Day, R. L., Laland, K. N., & Odling-Smee, F. J. (2003). Rethinking adaptation: The niche-construction perspective. *Perspectives in Biology and Medicine*, *46*, 80–95.

Dury, G. J., Moczek, A. P., & Schwab, D. B. (in review). Maternal and larval niche construction interact to shape development, survival, and population divergence in the dung beetle, *Onthophagus taurus*.

Dworkin, I. (2005). A study of canalization and developmental stability in the sternopleural bristle system of *Drosophila melanogaster*. *Evolution*, *59*, 1500–1509.

Emlen, D. J., Warren, I. A., Johns, A., Dworkin, I., & Lavine, L. C. (2012). A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science*, *337*, 860–864.

Emlen, D. J., Corley Lavine, L., & Ewen-Campen, B. (2007). On the origin and evolutionary diversification of beetle horns. *Proceedings of the National Academy of Sciences*, *104*, 8661–8668.

Estes, A. M., Hearn, D. J., Snell-Rood, E. C., Feindler, M., Feeser, K., Abebe, T., ... Moczek, A. P. (2013). Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLOS One*, *8*, e79061.

Fincher, G. T., & Woodruff, R. E. (1975). A European dung beetle, *Onthophagus taurus* Schreber, new to the U.S. (Coleoptera: Scarabaeidae). *Coleopterists Bulletin*, *29*, 349–350.

Flachowsky, G., & Hennig, A. (1990). Composition and digestibility of untreated and chemically treated animal excreta for ruminants-A review. *Biological Wastes*, *31*, 17–36.

Foray, V., Pérez-Jiménez, M. M., Fattouh, N., & Landmann, F. (2018). *Wolbachia* control stem cell behavior and stimulate

germline proliferation in filarial nematodes. *Developmental Cell*, *45*, 198–211.e3.

Frank, K., Brückner, A., Hilpert, A., Heethoff, M., & Blüthgen, N. (2017). Nutrient quality of vertebrate dung as a diet for dung beetles. *Scientific Reports*, *7*, 1–12.

Geminard, C., Arquier, N., Layalle, S., Bourouis, M., Slaidina, M., Delanoue, R., … Leopold, P. (2006). Control of metabolism and growth through insulin-like peptides in *Drosophila*. *Diabetes*, *55*, S5–S8.

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*, 372–375.

Gilbert, S. F. (2019). Developmental symbiosis facilitates the multiple origins of herbivory. *Evolution & Development*, 1–11. https://doi.org/10.1111/ede.12291

Gilbert, S. F., Bosch, T. C. G., & Ledón-Rettig, C. (2015). Eco-Evo-Devo: Developmental symbiosis and developmental plasticity as evolutionary agents. *Nature Reviews Genetics*, *16*, 611–622.

Gomez-Mestre, I., & Buchholz, D. R. (2006). Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the National Academy of Sciences*, *103*, 19021–19026.

Gotoh, H., Miyakawa, H., Ishikawa, A., Ishikawa, Y., Sugime, Y., Emlen, D. J., … Miura, T. (2014). Developmental link between sex and nutrition; doublesex regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. *PLOS Genetics*, *10*, e1004098.

Grimaldi, D., & Engel, M. S. (2005). *Evolution of the insects*. Cambridge, UK: Cambridge University Press.

Halffter, G., & Edmonds, W. D. (1982). The nesting behavior of dung beetles (Scarabaeinae). An ecological and evolutive approach (pp. 139–143). Mexico City, Mexico: Instituto de Ecologia.

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*, 25–41.

Holter, P. (2016). Herbivore dung as food for dung beetles: Elementary coprology for entomologists. *Ecological Entomology*, *41*, 367–377.

Hooper, L. V. (2001). Commensal host-bacterial relationships in the gut. *Science*, *292*, 1115–1118.

Hu, Y., Schmitt-Engel, C., Schwirz, J., Stroehlein, N., Richter, T., Majumdar, U., & Bucher, G. (2018). A morphological novelty evolved by co-option of a reduced gene regulatory network and gene recruitment in a beetle. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *285*, 20181373.

Ito, Y., Harigai, A., Nakata, M., Hosoya, T., Araya, K., Oba, Y., … Niimi, T. (2013). The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle. *EMBO Reports*, *14*, 561–567.

Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M., & Perlman, S. J. (2010). Adaptation via symbiosis: Recent spread of a *Drosophila* defensive symbiont. *Science*, *329*, 212–215.

Joos, B., Casey, T. M., Fitzgerald, T. D., & Buttemer, W. A. (1988). Roles of the tent in behavioral thermoregulation of eastern tent caterpillars. *Ecology*, *69*, 2004–2011.

Kijimoto, T., Costello, J., Tang, Z., Moczek, A. P., & Andrews, J. (2009). EST and microarray analysis of horn development in *Onthophagus beetles. BMC Genomics*, *10*, 504.

Kijimoto, T., Moczek, A. P., & Andrews, J. (2012). Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns. *Proceedings of the National Academy of Sciences United States of America*, *109*, 20526–20531.

Kijimoto, T., Pespeni, M., Beckers, O., & Moczek, A. P. (2012). Beetle horns and horned beetles: Emerging models in developmental evolution and ecology. *Wiley Interdisciplinary Reviews: Developmental Biology*, *2*, 405–418.

Koyama, T., Mendes, C. C., & Mirth, C. K. (2013). Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Frontiers in Physiology*, *4*, 263.

Kylafis, G., & Loreau, M. (2008). Ecological and evolutionary consequences of niche construction for its agent. *Ecology Letters*, *11*, 1072–1081.

Laland, K. N., Odling-Smee, F. J., & Feldman, M. W. (1999). Evolutionary consequences of niche construction and their implications for ecology. *Proceedings of the National Academy of Sciences United States of America*, *96*, 10242–10247.

Laland, K. N., Odling-Smee, J., & Gilbert, S. F. (2008). EvoDevo and niche construction: Building bridges. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *310*, 549–566.

Laland, K. N., Uller, T., Feldman, M., Sterelny, K., Müller, G. B., Moczek, A. P., … Odling-Smee, J. (2015). The extended evolutionary synthesis: its structure, assumptions, and predictions. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *282*, 20151019.

Ledón-Rettig, C. C., Pfennig, D. W., & Nascone-Yoder, N. (2008). Ancestral variation and the potential for genetic accommodation in larval amphibians: Implications for the evolution of novel feeding strategies. *Evolution & Development*, *10*, 316–325.

Levine, M., & Davidson, E. H. (2005). Gene regulatory networks for development. *Proceedings of the National Academy of Sciences United States of America*, *102*, 4936–4942.

Levis, N. A., Isdaner, A. J., & Pfennig, D. W. (2018). Morphological novelty emerges from pre-existing phenotypic plasticity. *Nature Ecology & Evolution*, *2*, 1289–1297.

Levis, N. A., & Pfennig, D. W. (2016). Evaluating 'plasticity-first' evolution in nature: Key criteria and empirical approaches. *Trends in Ecology & Evolution*, *31*, 563–574.

Li, Y., Brown, S. J., Hausdorf, B., Tautz, D., Denell, R. E., & Finkelstein, R. (1996). Two *orthodenticle*-related genes in the short-germ beetle *Tribolium castaneum*. *Development Genes and Evolution*, *206*, 35–45.

Linz, D. M., Hu, Y., & Moczek, A. P. (2019). The origins of novelty from within the confines of homology: the developmental evolution of the digging tibia of dung beetles. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *286*, 20182427.

Macagno, A. L. M., Beckers, O. M., & Moczek, A. P. (2015). Differentiation of ovarian development and the evolution of fecundity in rapidly diverging exotic beetle populations. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *323*, 679–688.

Macagno, A. L. M., Moczek, A. P., & Pizzo, A. (2016). Rapid divergence of nesting depth and digging appendages among tunneling dung beetle populations and species. *The American Naturalist*, *187*, E143–E151.

Macagno, A. L. M., Pizzo, A., Parzer, H. F., Palestrini, C., Rolando, A., & Moczek, A. P. (2011). Shape—but not size—codivergence between male and female copulatory structures in *Onthophagus* beetles. *PLOS One*, *6*, e28893.

Macagno, A. L. M., Zattara, E. E., Ezeakudo, O., Moczek, A. P., & Ledón-Rettig, C. C. (2018). Adaptive maternal behavioral plasticity and developmental programming mitigate the transgenerational effects of temperature in dung beetles. *Oikos*, *127*, 1319–1329.

McFall-Ngai, M. J. (2014). The importance of microbes in animal development: Lessons from the squid-vibrio symbiosis. *Annual Review of Microbiology*, *68*, 177–194.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences United States of America*, *110*, 3229–3236.

Moczek, A. P. (1998). Horn polyphenism in the beetle *Onthophagus taurus*: Larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. *Behavioral Ecology*, *9*, 636–641.

Moczek, A. P. (2003). The behavioral ecology of threshold evolution in a polyphenic beetle. *Behavioral Ecology*, *14*, 841–854.

Moczek, A. P. (2005). The evolution and development of novel traits, or how beetles got their horns. *BioScience*, *11*, 935–951.

Moczek, A. P. (2007). Developmental capacitance, genetic accommodation, and adaptive evolution. *Evolution & Development*, *9*, 299–305.

Moczek, A. P. (2009). Developmental plasticity and the origins of diversity: A case study on horned beetles. In T. N. Ananthakrishnan, & D. Whitman (Eds.), *Phenotypic plasticity in insects: mechanisms and consequences* (pp. 81–134). Plymouth, UK: Science Publishers, Inc.

Moczek, A. P. (2010). Phenotypic plasticity and diversity in insects. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *365*, 593–603.

Moczek, A. P. (2012). The nature of nurture and the future of evodevo: Toward a theory of developmental evolution. *Integrative and Comparative Biology*, *52*, 108–119.

Moczek, A. P., & Nijhout, H. F. (2002). Developmental mechanisms of threshold evolution in a polyphenic beetle. *Evolution & Development*, *4*, 252–264.

Moczek, A. P., & Nijhout, H. F. (2003). Rapid evolution of a polyphenic threshold. *Evolution & Development*, *5*, 259–268.

Moczek, A. P., Hunt, J., Emlen, D. J., & Simmons, L. W. (2002). Threshold evolution in exotic populations of a polyphenic beetle. *Evolutionary Ecology Research*, *4*, 587–601.

Muller, Z. O. (1980). Feed from animal wastes: State of knowledge, *FAO Animal Production and Health Paper 18*. Rome, Italy: Food and Agriculture Organization of the United Nations. http://www.fao.org/3/X6518E/X6518E00.htm

Odling-Smee, F. J., Laland, K. N., & Feldman, M. W. (2003). *Niche construction: The neglected process in evolution*. Princeton, NJ: Princeton University Press.

Paaby, A. B., & Rockman, M. V. (2014). Cryptic genetic variation: Evolution's hidden substrate. *Nature Reviews Genetics*, *15*, 247–258.

Parker, E. S., Dury, G. J., & Moczek, A. P. (2018). Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles. *Ecological Entomology*, *44*, 274–282.

Payne, J. L., Moore, J. H., & Wagner, A. (2014). Robustness, evolvability, and the logic of genetic regulation. *Artificial Life*, *20*, 111–126.

Posnien, N., Koniszewski, N. D. B., Hein, H. J., & Bucher, G. (2011). Candidate gene screen in the red flour beetle *Tribolium* reveals *six3* as ancient regulator of anterior median head and central complex development. *PLOS Genetics*, *7*, e1002416.

Posnien, N., Schinko, J. B., Kittelmann, S., & Bucher, G. (2010). Genetics, development and composition of the insect head—A beetle's view. *Arthropod structure & Development*, *39*, 399–410.

Prud'homme, B., Gompel, N., & Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proceedings of the National Academy of Sciences United States of America*, *104*, 8605–8612.

Queitsch, C., Sangster, T. A., & Lindquist, S. (2002). Hsp90 as a capacitor of phenotypic variation. *Nature*, *417*, 618–624.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). From the cover: Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences United States of America*, *101*, 4596–4601.

Robinson, B. W. (2013). Evolution of growth by genetic accommodation in Icelandic freshwater stickleback. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *280*, 20132197.

Rohner, N., Jarosz, D. F., Kowalko, J. E., Yoshizawa, M., Jeffery, W. R., Borowsky, R. L., … Tabin, C. J. (2013). Cryptic variation in morphological evolution: HSP90 as a capacitor for loss of eyes in cavefish. *Science*, *342*, 1372–1375.

Rutherford, S. L., & Lindquist, S. (1998). Hsp90 as a capacitor for morphological evolution. *Nature*, *396*, 336–342.

Saccone, G., Salvemini, M., Pane, A., & Polito, L. C. (2008). Masculinization of XX *Drosophila* transgenic flies expressing the *Ceratitis capitata* DoublesexM isoform. *The International Journal of Developmental Biology*, *52*, 1051–1057.

Sánchez, L., Gorfinkiel, N., & Guerrero, I. (2001). Sex determination genes control the development of the *Drosophila* genital disc, modulating the response to *Hedgehog*, *Wingless* and *Decapentaplegic* signals. *Development*, *128*, 1033–1043.

Schlichting, C. D., & Pigliucci, M. (1998). *Phenotypic evolution: A reaction norm perspective*. Sunderland, MA: Sinauer Associates Inc.

Schwab, D. B., & Moczek, A. P. (2017). Evo devo and niche construction. In Nuño de la Rosa, L., & Müller, G. B. (Eds.), *Evolutionary developmental biology—A reference guide* (pp. 1–14). Basel: Springer International Publishing.

Schwab, D. B., Casasa, S., & Moczek, A. P. (2017). Evidence of developmental niche construction in dung beetles: Effects on growth, scaling and reproductive success. *Ecology Letters*, *20*, 1353–1363.

Schwab, D. B., Casasa, S., & Moczek, A. P. (2019). On the reciprocally causal and constructive nature of developmental plasticity and robustness. *Frontiers in Genetics*, *9*, 735.

Schwab, D. B., Flores, B. J., Linz, D., Moczek, A. P., & Tennessen, J. M. (In preparation). Characterizing the developmental and metabolic response to hypoxia in dung beetles.

Schwab, D. B., Riggs, H. E., Newton, I. L. G., & Moczek, A. P. (2016). Developmental and ecological benefits of the maternally transmitted microbiota in a dung beetle. *The American Naturalist*, *188*, 679–692.

Scoville, A. G., & Pfrender, M. E. (2010). Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators.

Proceedings of the National Academy of Sciences United States of America, *107*, 4260–4263.

Shaw, K. A., Scotti, M. L., & Foster, S. A. (2007). Ancestral plasticity and the evolutionary diversification of courtship behaviour in threespine sticklebacks. *Animal Behaviour*, *73*, 415–422.

Shikuma, N. J., Pilhofer, M., Weiss, G. L., Hadfield, M. G., Jensen, G. J., & Newman, D. K. (2014). Marine tubeworm metamorphosis induced by arrays of bacterial phage tail-like structures. *Science*, *343*, 529–533.

Shingleton, A. W., Das, J., Vinicius, L., & Stern, D. L. (2005). The temporal requirements for insulin signaling during development in *Drosophila*. *PLOS Biology*, *3*, e289.

Shubin, N., Tabin, C., & Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. *Nature*, *457*, 818–823.

Shukla, S. P., Sanders, J. G., Byrne, M. J., & Pierce, N. E. (2016). Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular Ecology*, *25*, 6092–6106.

Sikkink, K. L., Reynolds, R. M., Ituarte, C. M., Cresko, W. A., & Phillips, P. C. (2014). Rapid evolution of phenotypic plasticity and shifting thresholds of genetic assimilation in the nematode *Caenorhabditis remanei*. *G3: Genes Genomes Genetics*, *4*, 1103–1112.

Silva, D. P., Vilela, B., Buzatto, B. A., Moczek, A. P., & Hortal, J. (2016). Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, *18*, 3137–3148.

Sneed, J. M., Sharp, K. H., Ritchie, K. B., & Paul, V. J. (2014). The chemical cue tetrabromopyrrole from a biofilm bacterium induces settlement of multiple Caribbean corals. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, 281, 1–9.

Snell-Rood, E. C., Burger, M., Hutton, Q., & Moczek, A. P. (2016). Effects of parental care on the accumulation and release of cryptic genetic variation: Review of mechanisms and a case study of dung beetles. *Evolutionary Ecology*, *30*, 251–265.

Sole, C. L., & Scholtz, C. H. (2010). Did dung beetles arise in Africa? A phylogenetic hypothesis based on five gene regions. *Molecular Phylogenetics and Evolution*, *56*, 631–641.

Sommer, F., & Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology*, *11*, 227–238.

Standen, E. M., Du, T. Y., & Larsson, H. C. E. (2014). Developmental plasticity and the origin of tetrapods. *Nature*, *513*, 54–58.

Suzuki, Y., & Nijhout, H. F. (2006). Evolution of a polyphenism by genetic accommodation. *Science*, *311*, 650–652.

Tanaka, K., Barmina, O., Sanders, L. E., Arbeitman, M. N., & Kopp, A. (2011). Evolution of sex-specific traits through changes in HOX-dependent *doublesex* expression. *PLOS Biology*, *9*, e1001131.

Tang, H. Y., Smith-Caldas, M. S. B., Driscoll, M. V., Salhadar, S., & Shingleton, A. W. (2011). *FOXO* regulates organ-specific phenotypic plasticity in *Drosophila*. *PLOS Genetics*, *7*, e1002373.

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLOS Biology*, *6*, e1000002.

Tomoyasu, Y., Ohde, T., & Clark-Hachtel, C. (2017). What serial homologs can tell us about the origin of insect wings. *F1000Research*, *6*, 268.

Tyndale-Biscoe, M. (1996). *Australia's introduced dung beetles: original releases and redistributions*. Technical Report 62. Canberra, ACT: CSIRO, Division of Entomology.

Uller, T., Moczek, A. P., Watson, R. A., Brakefield, P. M., & Laland, K. N. (2018). Developmental bias and evolution: A regulatory network perspective. *Genetics*, *209*, 949–966.

von Dassow, G., Meir, E., Munro, E. M., & Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature*, *406*, 188–192.

Waddington, C. H. (1953). Genetic assimilation of an acquired character. *Evolution*, *7*, 118–126.

Wagner, A. (2005). *Robustness and evolvability in living systems*. Princeton, NJ: Princeton University Press.

Wagner, G. P. (2007). The developmental genetics of homology. *Nature Reviews Genetics*, *8*, 473–479.

Wagner, G. P. (2014). *Homology, Genes, and Evolutionary Innovation*. Princeton, NJ: Princeton University Press.

Walworth, N. G., Lee, M. D., Fu, F.-X., Hutchins, D. A., & Webb, E. A. (2016). Molecular and physiological evidence of genetic assimilation to high CO_2 in the marine nitrogen fixer *Trichodesmium*. *Proceedings of the National Academy of Sciences United States of America*, *113*, E7367–E7374.

West-Eberhard, M. J. (1986). Alternative adaptations, speciation, and phylogeny (A Review). *Proceedings of the National Academy of Sciences United States of America*, *83*, 1388–1392.

West-Eberhard, M. J. (1998). Evolution in the light of developmental and cell biology, and vice versa. *Proceedings of the National Academy of Sciences United States of America*, *95*, 8417–8419.

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. New York, NY: Oxford University Press.

Whalan, S., & Webster, N. S. (2014). Sponge larval settlement cues: The role of microbial biofilms in a warming ocean. *Scientific Reports*, *4*, 28–32.

Wund, M. A., Baker, J. A., Clancy, B., Golub, J. L., & Foster, S. A. (2008). A test of the "flexible stem" model of evolution: Ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. *The American Naturalist*, *172*, 449–462.

Yeh, P. J., & Price, T. D. (2004). Adaptive phenotypic plasticity and the successful colonization of a novel environment. *The American Naturalist*, *164*, 531–542.

Zattara, E. E., Busey, H. A., Linz, D. M., Tomoyasu, Y., & Moczek, A. P. (2016). Neofunctionalization of embryonic head patterning genes facilitates the positioning of novel traits on the dorsal head of adult beetles. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *283*, 20160824.

Zattara, E. E., Macagno, A. L. M., Busey, H. A., & Moczek, A. P. (2017). Development of functional ectopic compound eyes in scarabaeid beetles by knockdown of *orthodenticle*. *Proceedings of the National Academy of Sciences United States of America*, *114*, 12021–12026.

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CURRICULUM VITAE

Erik Parker

915 East Third Street
Bloomington, IN 47405
https://erikpark.github.io
esparker7@gmail.com

EDUCATION

2015 - 2021: Ph.D. Evolution, Ecology and Behavior, Indiana University, Bloomington
Minor: Statistics
Advisor: Dr. Armin Moczek
Committee: Dr. Irene Newton, Dr. Jay Lennon, Dr. Whitney Schlegel, Dr. Jen Lau
Thesis title: *With a little help from my friends: The role of the microbiota in dung beetle diversification*.

2009 – 2014: Bachelor of Science, Biology, University of Oregon, Eugene; *Magna Cum Laude*

PUBLICATIONS [*Co-First Authors]

in press / in print

2021 **Parker ES,** Moczek AP, Macagno ALM. Reciprocal microbiome transplants differentially rescue fitness in two syntopic dung beetle sister species (Scarabaeidae: *Onthophagus*). **Ecological Entomology**.

2020 **Parker ES**, Moczek AP. Don't stand so close to me: microbiota-facilitated enemy release dynamics in introduced *Onthophagus taurus* dung beetles. **Ecology and Evolution**.

Parker ES, Newton ILG, Moczek AP. (My microbiome) would walk 10,000 miles: Maintenance and turnover of microbial communities in introduced dung beetles. **Microbial Ecology**.

*Hu Y, *Linz DM, ***Parker ES**, *Schwab DB, Casasa S, Macagno AL, Moczek AP. Developmental bias in horned dung beetles and its contributions to innovation, adaptation, and resilience. **Evolution & Development** 22(1-2):165-80.

2019 **Parker ES**, Dury GJ, Moczek AP. Transgenerational developmental effects of species-specific, maternally transmitted microbiota in Onthophagus dung beetles. **Ecological Entomology** 44(2):274-82.

PRESENTATIONS

2019 **Parker ES**, Schwab DB & Moczek AP. With a little help from my friends: The role of the microbiota in dung beetle diversification. [poster] *Evolution Evolving*, Cambridge, UK.

Parker ES. Where does diversity come from? Symbiosis, development, and evolution of dung beetles. *Brown Bag Seminar*. Indiana University, Bloomington, IN.

2014 **Parker ES**, Currey M & Cresko W. Morphological Divergence Between Coastal and Inland Freshwater Oregon Stickleback. *EVO-WIBO*, Port Townsend, WA.

GRANTS, AWARDS, AND HONORS

2019 Louise Constable Hoover Fellowship ($2000 for research expenses). Indiana University, Bloomington.

Indiana University McCormick Science Grant ($2500 for research expenses). Indiana University, Bloomington.

2017 EEB Summer Fellowship ($3200 stipend). Indiana University, Bloomington.

2016 NSF Graduate Research Fellowship Program (Honorable Mention). *A test of the hologenome theory of evolution in natural populations.*

TEACHING EXPERIENCE

2018 Assistant Instructor. Animal Behavior 460. Indiana University, Bloomington.

2017 Instructor, Foundations in Science and Mathematics program. Zoology. Bloomington, IN.

Assistant Instructor. Biology of the Senses 104. Indiana University, Bloomington.

Assistant Instructor. Diversity, Evolution, and Ecology 111. Indiana University, Bloomington.

2016 Guest Lecture. Diversity, Evolution, and Ecology 111. *IU's Finest: Natural Selection in the Classroom*. Indiana University, Bloomington.

Guest Lecture. Evolution 318. *Evolution and Development: The Past, Present, and Future of EvoDevo*. Indiana University, Bloomington.

- continued -

2016 Assistant Instructor. Evolution 318. Indiana University, Bloomington.

Assistant Instructor. Entomology 373. Indiana University, Bloomington.

2015 Guest Lecture. Evolution 318. *Evolution and Development: The Past, Present, and Future of EvoDevo*. Indiana University, Bloomington.

Assistant Instructor. Evolution 318. Indiana University, Bloomington.

Assistant Instructor. Human Biology 104. Indiana University, Bloomington.

WORKSHOPS

LEAD

2020 *Making beautiful (or at least decent looking) scientific graphics using Illustrator*. Indiana University, Bloomington.

Using R for data visualization, statistical analyses, and linear modeling of biological data. Indiana University, Bloomington.

PARTICIPANT

2018 STEM Tech2Teach. Organizers: Dr. Madeleine Gonin and Dr. John Paul Kanwit. Center for Innovative Teaching and Learning, Indiana University, Bloomington.

2017 *GitHub and R for Ecologists*. Organizer: Dr. Jay Lennon. Indiana University, Bloomington.

The Basics of *Genomics and Bioinformatics*. Organizer: Dr. Irene Newton. Indiana University, Bloomington.

PROFESSIONAL SERVICE

Invited Reviewer for: *Entomologia Experimentalis et Applicata*,
Evolution & Development.

STUDENTS MENTORED

2018- 2019 Madison Gits (Undergraduate)

OUTREACH

2019 *The Evolutionary Morphology of Skulls and Teeth*. 6 classes of 9th-12th graders. Mooresville High School, Mooreseville, IN. Participants: ~ 180 students.

2018 *Studying Animal Behavior in Crayfish*. 4 classes of AP Biology students. Bloomington High School South, Bloomington, IN. Participants: ~80 students.

The Wonderful World of Dung Beetles. Community outreach event. Marble Hill Farms, Bloomington, IN. Participants: ~20 children and adults.

The Evolutionary Morphology of Skulls and Teeth. 7 classes of 9th-12th graders. Mooresville High School, Mooreseville, IN. Participants: ~ 220 students.

Reconstructing 4 Million Years Human Evolution. 1 class of 9th-12th graders. Harmony School, Bloomington, IN. Participants: ~15 students.

2017 *Insect Lifecycles*. 2 classes of 1st and 2nd graders. Parkview Primary School, Bedford, IN. Participants: ~70 students.

The Evolutionary Morphology of Skulls and Teeth. 2 classes of 9th-12th graders. Bloomington High School South, Bloomington, IN. Participants: ~ 50 students.

2016 *The Evolutionary Morphology of Skulls and Teeth*. 5 classes of 9th-12th graders. Bloomington High School North, Bloomington, IN. Participants: ~ 160 students.

Reconstructing 4 Million Years Human Evolution. 4 classes of 6th-8th graders. Central Middle School, Columbus, IN. Participants: ~100 students.

The Evolutionary Morphology of Skulls and Teeth. 3 classes of 3rd-5th graders. Unionville Elementary School, Unionville, IN. Participants: ~ 70 students.

Formulating and Testing Hypotheses Using Termite Behavior. 3 classes of 1st-3rd graders. Unionville Elementary School, Unionville, IN. Participants: ~ 80 students.

The Evolutionary Morphology of Skulls and Teeth. 1 class of 6th graders. Summit Elementary School, Bloomington, IN. Participants: ~ 30 students.

2015 *The Wonderful World of Dung Beetles*. WonderLab camp, grades K-3rd. Bloomington, IN. Participants: ~40 students.

The Evolutionary Morphology of Skulls and Teeth. 7 classes of 9th-12th graders. Bloomington High School South, Bloomington, IN. Participants: ~ 210 students.

The Evolutionary Morphology of Skulls and Teeth. 4 classes of 2nd-5th graders. Dollens Elementary School, Oolitic, IN. Participants: ~ 120 students.

Judge for 2015 *Indiana Science Olympiad Tournament*. Indiana University, Bloomington, IN.

2014 *The Evolutionary Morphology of Skulls and Teeth*. 4 classes of 4th-8th graders. St. Vincent de Paul School, Bedford, IN. Participants: ~ 80 students.

The Evolutionary Morphology of Skulls and Teeth. Community outreach event. Bedford Public Library, Bedford, IN. Participants: ~ 10 children and adults.

Insect Development and Biodiversity. 1 class of students age 5-18. Bloomington Christian Schoolhouse, Bloomington, IN. Participants: 35 students.

The Evolutionary Morphology of Skulls and Teeth. 2 classes of 5th graders. Spencer-Owen Elementary School, Spencer, IN. Participants: ~ 50 students.

2013 *Boology! A Night of Spooky Science.* Community outreach event. University of Oregon, Eugene, OR. Participants: ~ 500 visitors.